BIOCHEMICAL AND IMMUNOLOGICAL STUDIES ON REACTIVE OXYGEN MODIFIED GLYCATED HUMAN SERUM ALBUMIN-POSSIBLE IMPLICATIONS IN DIABETES MELLITUS

THESIS SUBMITTED FOR THE DEGREE OF

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IN

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BY

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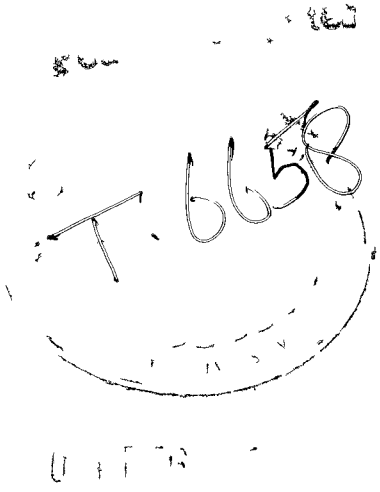

Dedicated To My Father

(Who gave me roots for support and wings to fly high)

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CERTIFICATE

I certify that the work entitled ***"Immunological and Biochemical studies on Reactive Oxygen Modified Glycated Human Serum Albumin-Possible Implications in Diabetes Mellitus"*** embodied in this thesis is an original work carried out independently by **Mr. Mohd. Wajid Ali Khan** at the Department of Biochemistry, J.N. Medical College, Aligarh Muslim University, Aligarh, under the supervision of Prof. Rashid Ali and is suitable for the award of **Ph.D.** degree in **Biochemistry**.

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CONTENTS

ABSTRACT

Non-enzymatic glycation and oxidation play an important role in the pathogenesis of several diseases like diabetes and rheumatoid arthritis (Newkirk *et al.*, 2003; Jakus, 2003; Schmitt *et al.*, 2005). They also induce the accelerated accumulation of AGE products in tissues of diabetic patient, particularly with secondary complications like retinopathy, nephropathy and artherosclerosis (Cohen *et al.*, 2005; Defraigne, 2005). In diabetes mellitus and rheumatoid arthritis protein glycation and the formation of AGEs are accompanied by increased free radical activity that contributes toward the biomolecular damage (Ahmed, 2005; Drinda *et al.*, 2005; Sunahori *et al.*, 2006). AGE formation is an inevitable process *in vivo* and can be accelerated under pathological conditions such as oxidative stress. Oxidative stress and oxidative damage to tissues are common end points of chronic diseases such as diabetes and rheumatoid arthritis (Baynes and Thorpe, 1999; Ahmed *et al.*, 2005).

In the present study, human serum albumin (HSA) was glycated non-enzymatically by incubating with glucose for 10 weeks. Glycated HSA was further modified by ROS generated by the irradiation of hydrogen peroxide with UV light at 254 nm. Under these experimental conditions, the major species would be hydroxyl radical ($^{\cdot}OH$), the most reactive of ROS. The modified HSA samples showed remarkable biophysical changes analyzed by gel electrophoresis, spectral analysis, circular dichroism spectropolarimetry and thermal denaturation studies. Estimation of ketoamine, carbonyl and free amino groups revealed that glycation and oxidation attributed to the conformational and structural changes in HSA.

The electrophoretic pattern of both modified HSA showed formation of high and low molecular weight aggregates. However, ROS-glycated HSA showed almost similar changes but with considerable decrease in their intensity which can be attributed to fragmentation of glycated HSA on ROS modification. UV spectra of glycated HSA showed hypochromicity, whereas, ROS-glycated HSA exhibited hyperchromicity as compared to unmodified HSA. These changes are indicative of conformational changes in HSA on modifications. Glycation causes shielding of aromatic amino acids contributing to hypochromicity. However, fragmentation by $^{\cdot}OH$ causes exposure of aromatic amino acids in glycated HSA towards solvent

system resulting in hyperchromicity. The fluorescence spectral studies of HSA also showed the same pattern as observed in UV spectra as the same aromatic amino acids are involved in both spectral analyses.

Tryptophan specific fluorescence was also carried out. Glycated HSA showed lower fluorescence intensity and quantum yield, as compared to ROS glycated HSA showing higher tryptophan specific fluorescence and quantum yield. The significant observation in this result was the blue shift on modifications. The blue shift was more in case of glycated HSA than ROS-glycated HSA. The results reiterate the earlier observation of conformational changes in glycated HSA whereas fragmentation appears to be one of prominent phenomena on ROS exposure.

The melting temperature profile of glycated and ROS-glycated HSA showed a net increase of 18.1°C and 8.8°C, respectively, as compared to unmodified protein. Once again it can be attributed to conformational stability of both modified HSA over their native form. However, ROS may causes disruption of weak bonds or fragmentation of glycated HSA and might be one of the reasons for increase in Tm value.

Furthermore, colorimetric estimations were carried out to support the biophysical analysis. Ketoamine level was found to be significantly higher in case of both modified samples (with slight difference) as compared to native HSA, which showed negligible ketoamine content. Levels of carbonyl groups were also elevated in both cases, an important marker of both glycation and oxidative stress. However, the increase was more in ROS modified glycated HSA. Number of free amino groups in modified HSA samples was found to be half as compared to native HSA. ROS modification of glycated HSA resulted in appreciable increase in amino groups reiterating once again the fragmentation and structural changes in $^{\bullet}OH$-modified glycated polymer.

Studies with various antioxidants, scavengers and metal chelators showed inhibition of different parameters in both the modified samples. Moreover, maximum inhibition with aminoguanidine and combination of two enzymatic antioxidant

(catalase and SOD) was observed showing a definite role of ROS in the modification of glycated HSA and AGE formation.

Native and modified HSA samples were used to induce antibodies in rabbits and were found to be immunogenic, producing high titer antibodies. The antigenic specificity of the induced antibodies was studied by direct binding ELISA, immunodiffusion and competition ELISA. The immunogen showed a high degree of specificity for the induced antibodies, reiterated by gel retardation assay. Anti-glycated and ROS-glycated HSA antibodies showed preferential recognition of glycated and ROS-glycated HSA in a competition assay. The induced antibodies were polyspecific in nature.

Sera of diabetic patients were tested for the presence of antibodies reactive with native and both the modified samples of HSA. Direct binding ELISA showed greater recognition for modified HSA samples as compared to the native form ascertained by competition ELISA. Moreover, significantly higher recognition of modified HSA was observed in the sera of diabetic patients having secondary complications like retinopathy, nephropathy and artherosclerosis. The higher binding to both modified HSA over native HSA of antibodies in the sera of diabetic patients suggests the involvement of modified HSA in the production of autoantibodies in these patients. The binding specificity of glycated and ROS-glycated HSA with diabetic patient's IgG was reiterated by gel retardation assay.

Glycation and ROS damage to human blood proteins was detected immunochemically using anti-glycated and anti-ROS-glycated HSA antibodies as probes. Albumin and IgG from different diabetic patients inhibited antibodies binding to their respective immunogen demonstrating the presence of glycated and ROS-glycated epitopes on albumin and IgG molecules. Data obtained from our studies correlates to the earlier studies that glycation and glycoxidation causes *in vivo* protein modifications.

The binding of circulating autoantibodies in rheumatoid arthritis with native, glycated and ROS-glycated HSA was also analyzed. Direct binding ELISA results showed preferential binding of rheumatoid arthritic autoantibodies to both modified

HSA in comparison to native HSA. Inhibition ELISA reiterated the direct binding results. Gel retardation assay further substantiated the binding of both modified HSA with rheumatoid arthritic autoantibodies.

In conclusion, glycation and oxidation causes damage to HSA and render it highly immunogenic. Polyclonal antibodies generated against modified antigens showed preferential recognition of the immunogens. The induced antibodies as immunochemical probes detected glycation and oxidative damage to the blood proteins. Recognition of modified HSA samples by antibodies in sera of patients with diabetes and rheumatoid arthritis suggests glycation and oxidation induced blood proteins damage in these patients. It is, therefore, postulated that glycation and ROS modification of blood proteins appears to play a major role in the production of autoantibodies in disease state(s).

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ABBREVIATIONS

A_{280}	: Absorbance
AGEs	: Advance glycation end products
BPB	: Bromophenol blue
BSA	: Bovine serum albumin
CD	: Circular dichroism
DNA	: Deoxyribonuleic acid
ELISA	: Enzyme linked immunosorbent assay
HSA	: Human serum albumin
IC	: Immune complex
IgG	: Immunoglobulin G
ID	: Immunodiffusion
MW	: Molecular weight
NBT	: Nitrobluetetrazolium
OD	: Optical density
PBS	: Phosphate buffer saline
ROS	: Reactive oxygen species
SDS-PAGE	: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOD	: Superoxide dismutase
TBS	: Tris buffer saline
TEMED	: N.N.N.N-tetracthylmethylene diamine
Tm	: Melting temperature
TNBS	: Trinitrobenzenesulphonic acid
TRIS	: Tris (hydroxylmethyl) amino methane

UV	: Ultraviolet
λ_{max}	: Wavelength showing maximum absorbance
nm	: Nanometer
µl	: Microlitre
µg	: Microgram
mg	: Milligram
ml	: Milliliter
v/v	: volume by volume
w/w	: weight by weight

INTRODUCTION

As the name indicates proteins are of paramount importance for biological systems. Out of total dry body weight, $3/4^{th}$ is made up of proteins. Proteins are like building blocks i.e. all the major structure and functional aspects of the body are carried out by protein molecules. All proteins are polymers of amino acids. Commonly occurring amino acids are 20 in number.

It is almost impossible to correctly classify all proteins. However, proteins may be divided into three major groups; simple, conjugated and derived. Simple protein includes albumin, globulin, protamines, lectins and scleroproteins. Conjugated protein contains glycoproteins, lipoproteins, chromoproteins, phosphoproteins and metalloproteins. Derived proteins are degraded products of the native proteins (Vasudevan and Sreekumari, 2000).

Human serum albumin

Serum albumin belongs to a multigene family of proteins that includes α-fetoprotein (AFP) and human group-specific component (GC). It is relatively large multi-domain protein, which, as the major soluble protein constituents of the circulatory system, has many physiological functions and has a half life of about 20 days (Ancell, 1939).

Originally, the protein was referred to as "albumen" derived from latin word *albus* meaning "white", after the white colour of flocculant precipitate produced by various proteins. About 40% of the total albumin is found in the circulatory plasma (Peters, 1970) where as of the remaining 60%, about half resides in viscera and half in muscle and skin (Rabilloud *et al.*, 1988). Albumin also occurs in milk (Phillippy and McCarty, 1979), amniotic fluid (Bala *et al.*, 1987), semen (Blumsohn *et al.*, 1991) and mammary cyst (Balbin *et al.*, 1991).

Albumin concentration in plasma declines slightly with age (Cooper and Gardner, 1989) but it is lower in new borns (Cartlidge and Ruter, 1986) and as low as 20.0 g/l in premature infants (Reading *et al.*, 1990). Albumin is produced by the liver at the rate of 0.7 mg/g liver per hr (Peters, 1985). About 4-5% of albumin is daily replaced by hepatic synthesis (Olufemi *et al.*, 1990). Albumin concentration in plasma is maintained through transcriptional control of the albumin gene by the anabolic hormones-insulin and somatotrophin (Hudson *et*

al., 1987). Albumin synthesis is also highly dependent upon the supply of dietary amino acids (Kaysen *et al.*, 1989).

The first crystal structure of HSA at low resolution was reported by Carter and coworkers in 1989 (Carter *et al.*, 1989; Carter and He, 1990), and its refined structure at 2.8 Å resolution was published by the same group. The crystal structure of HSA shows that it is a three-domain, each domain contain two sub domains, heart shaped molecule that is predominantly composed of helical structure, with the remaining polypeptides in turns and extended or flexible regions that connect subdomains. Each domain is composed of two sub-domains that are stabilized by internal disulphide bridges (He and Carter, 1992). Albumin have 585 amino acids, this globular M_r 65 kDa protein contains 18 tyrosines, six methionines, one tryptophan, 17 disulphide bridges, and only one free cysteine, Cys34 (Sugio *et al.*, 1999). Roughly 67% of HSA is helical, with the remainder in turns and extended polypeptide. The disulphide pairings in the primary structure of HSA predicted by Brown (Brown *et al.*, 1989) (Fig. 1).

Functions of human serum albumin

Albumin contributes to about 80% of colloid blood pressure (Lundsgaard, 1986) and 100% of protein effect in the acid base balance of plasma (Figge *et al.*, 1991). It acts as a carrier for long chain fatty acid (Brodersen *et al.*, 1991; Cistola and Small, 1991), their acyl-coenzyme esters (Richards *et al.*, 1990) and monoacyl phospholipids (Robinson *et al.*, 1989) and affects the activity of lipase, esterases (Posner *et al.*, 1987) and carnitine acyl transferase (Richards, 1991). Albumin binds to polyunsaturated fatty acids (Anel *et al.*, 1989) and influences the stability (Haeggstrom *et al.*, 1983), biosynthesis (Heinsohn *et al.*, 1987) and transformations of prostaglandins (Dieter *et al.*, 1990). It binds weakly to cholesterol (Deliconstantinos *et al.*, 1986), bile acids (Malavolti *et al.*, 1989), corticoid hormones (Watanabe *et al.*, 1991; Mendel *et al.*, 1990) sex hormone (Padridge, 1988), thyroxine (Petitpas *et al.*, 2003) and is involved in transport of thyroid hormones (Mendel *et al.*, 1990). Albumin also helps in the transport of

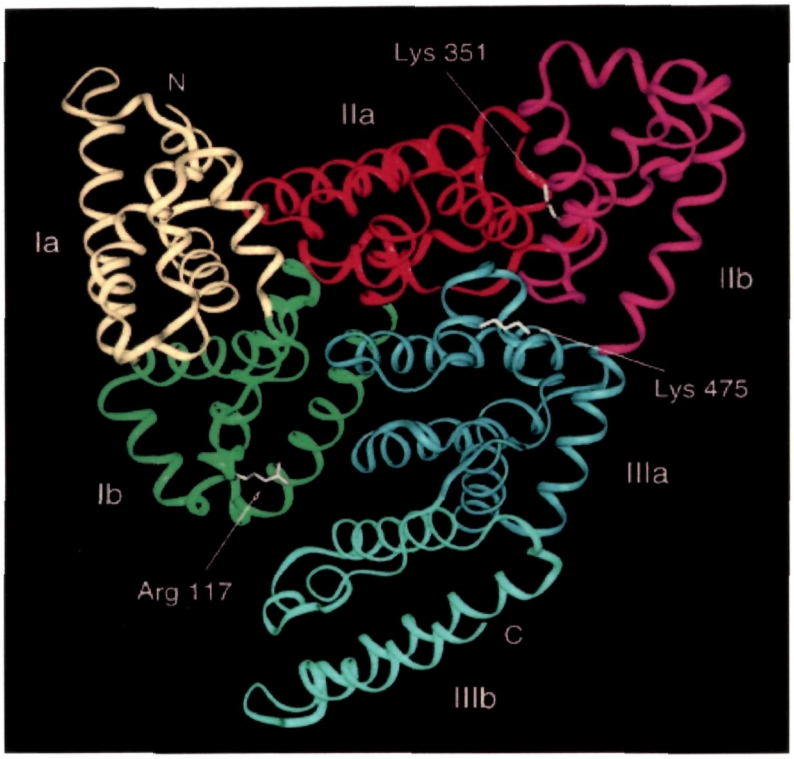


Fig. 1. Schematic drawing of the HSA molecule. Each subdomain is marked with a different color (yellow for subdomain Ia; green, Ib; red, IIa; magenta, IIb; blue, IIIa; and cyan, IIIb). N- and C-termini are marked as N and C, respectively. Arginine 117, lysine 351 and lysine 475, which may be binding sites for long-chain fatty acids, are colored white (Sugio *et al.*, 1999).

pyridoxal phosphate (Fonda *et al.*, 1991), cystein and glutathione (Joshi *et al.*, 1987) by forming a covalent bond with these ligands.

Albumin is also responsible for the transport and store housing of many therapeutic drugs in the blood stream (Bhattacharya *et al.*, 2000). It is an important constituent of tissue culture media (Barnes and Sato, 1980) and serves as a medium to support the growth of bacteria, fungi and yeast (Callister *et al.*, 1990; Morrill *et al.*, 1990).

Albumin is also proposed to serve high affinity to metals such as Cu^{2+} and Zn^{2+} and act as an antioxidant function in the vascular compartment because of its scavenging of reactive oxygen and nitrogen species that are generated by basal aerobic metabolism and can be produced at increased rates during inflammation (Halliwell, 1988; Halliwell and Gutteridge, 1990).

The anticoagulant and antithrombotic effects of albumin are poorly understood, this may be due to binding nitric oxide radicals inhibiting inactivation and permitting a more prolonged anti-aggregatory effect.

It is possible that albumin has a role in limiting the leakage from capillary beds during stress that increases capillary permeability. This can be related to the ability of endothelial cells to control the permeability of their walls, and the spaces between them (Jakus and Rietbrock, 2004).

Antigenecity of human serum albumin

Albumin is highly antigenic giving high antibody titers in experimental animals due to which it was considered a popular source of antigen by immunologists' way before the development of precipitation reaction demonstrating molar ratio of bovine serum albumin (BSA) to antibody at equivalence point of 4:1 (Heidelberger, 1938). Recent studies, suggests that there are 13 major antigenic sites on HSA which are recognized by 19 monoclonal antibodies (Doyen *et al.*, 1985) which is consistent with the maximum number of sites predicted by precipitation analysis thus indicating a third of the surface of albumin being antigenic. The synthetic antigenic sites localized with rabbit antisera against bovine serum albumin, were shown to bind considerable amounts

of specific mouse 125 I-labelled antibodies against bovine serum albumin, this showed that recognition of the antigenic sites of serum albumin is independent of the immunized species and is inherent in their structural and conformational uniqueness (Sakate and Atassi, 1980). Albumin-specific antibodies could also be used for albumin quantifications not only in urine, but also in other biological fluids. Anti-albumin monoclonoal antibodies could be used for the preparation of immunosorbents to deplete albumin from serum, which is a necessary stage of proteomic studies of blood proteins. A-HAlb/98 hybridoma secreting monoclonal antibody that specifically recognizes HSA, the ability of these antibodies is to quantitate urinary albumin in nanogram ranges (Aybay *et al.*, 1999: Aybay and Karakus, 2003).

IgG reactivity towards glycated HSA was significantly higher in diabetic than in control sera (Vay *et al.*, 2000). Certain sera, IgG, IgM, IgA and IgE antibodies against formaldehyde-HSA could be measured. In the highest titered sera, it was shown that the IgG antibody was not directed against formaldehyde (F) alone or F-lysine but against an antigenic grouping of F-HSA. Some sera from dialysis patients had antibody activity against HSA. Two individuals with a history of F-induced asthma had no IgG antibodies but did have IgE antibodies against F-HSA and HSA (Fiehn *et al.*, 2004).

HSA specific polyclonal rabbit immunoglobulin was labelled with biotin and then used as the tracer antibody makes it a favourable candidate for utilization in diagnostic applications as well as in research studies (Aybay and Karakus, 2003).

Non-enzymatic glycation of proteins

Non-enzymatic glycation is a process by which sugar is chemically bound to free ε-amino groups of proteins but without the help of enzymes. However, in the recent years extensive investigations have been made on the glycation of proteins exposed directly to high glucose concentrations, e.g. lens crystalline proteins (Stevvens *et al.*, 1978), insulin (Dolhofer and Wieland, 1979), proteins of erythrocyte membrane (Miller *et al.*, 1980), bovine serum albumin (Arakawa and Timasheff, 1982), human serum albumin (Shaklai *et al.*, 1984), enzymes

(Coradello *et al.*, 1984), high and low-density lipoproteins (Krittein *et al.*, 1990), peripheral nerve mylin (Green, 1980), elastin (Baydanoff *et al.*, 1994) and immunoglobulin G (Newkirk *et al.*, 2003).

It is a classical covalent reaction in which, by means of N-glycoside bonding, the sugar-protein complex is formed through a series of chemical reactions described by a chemist Maillard (Singh *et al.*, 2001). Maillard reactions are complex and multilayer, and can be analyzed in three steps. (i) The sugar-protein complex is formed first (Amadori rearrangement), an early product of non-enzymatic glycation lead to an intermediary products which is a precursor of all later compounds. (ii) It includes the formation of numerous intermediary products, some of which are very reactive and continue with glycation reaction. (iii) Final phase consists of polymerization reaction of the complex products formed in the second step, whereby heterogeneous structures named advanced glycation end products (AGE) are formed (Fig. 2). It was believed that the primary role in Maillard reactions was exclusively played by high glucose concentration. However, recent data show that, in spite of the fact that sugars are the main precursors of AGE compounds, numerous intermediary metabolites, i.e. α-oxoaldehydes, also creatively participate in non-enzymatic glycation reactions. Such intermediary products are generated during glycolysis (methylglyoxal) or along the polyolic pathway, and can also be formed by autoxidation of carbohydrates (glyoxal).

Advanced glycation end products

Protein modification with AGE is irreversible, as there are no enzymes in the body that would be able to hydrolyze AGE compounds. These structures then accumulate during the lifespan of the protein on which they have been formed. Examples include all types of collagen, albumin, basic myelin protein, eye lens proteins, lipoproteins, and nucleic acid. It is now well documented that AGE change the function of many proteins, thus contributing to various late complications of diabetes mellitus (Turk, 1997). The major biological effects of excessive glycation include: inhibition of regulatory molecule binding, crosslinking of glycated proteins, trapping of soluble proteins by glycated

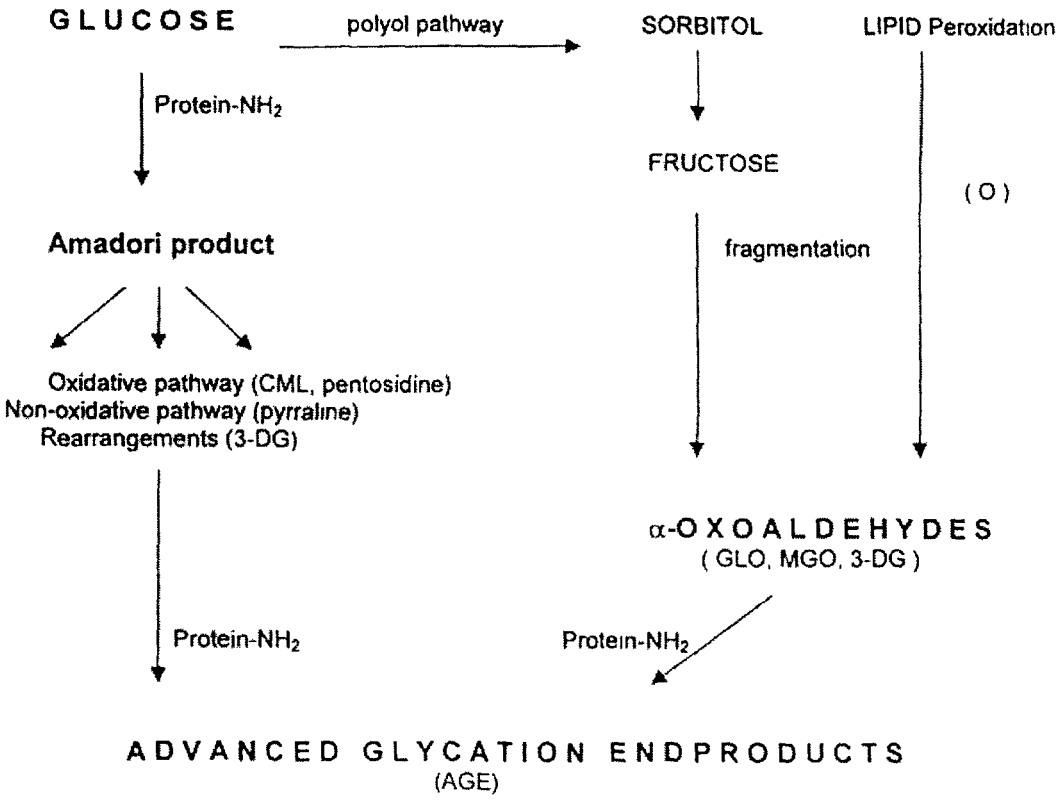


Fig. 2. Schematic representation of potential pathway leading to AGE formation. The abbreviations given above are represented as, GLO=glyoxal; MGO methylglyoxal; 3-DG 3-deoxyglucosone; CML=carboxymethyl-lysine (Turk, 2001).

extracellular matrix, decreased susceptibility to proteolysis, inactivation of enzymes, abnormalities of nucleic acid function, and increased immunogenicity in relation to immune complex formation (Turk, 2001).

Glycotoxins (AGE peptides)

In process of glycation, AGE peptides are released as degradation products, which partly occur through proteolysis of the matrix component commonly named glycotoxins. Glycotoxins (AGE peptides) are very reactive on entering blood circulation. In case they have not been eliminated through the kidneys, recirculating AGE peptides can generate new AGE products that react with other plasma or tissue components. At this stage, glycation becomes an autonomic process, which significantly accelerates the progress of the complication (Turk, 2001).

Glycation of human serum albumin

Serum albumin non-enzymatically reacts with glucose yielding a stable glycated form of albumin (Garlick and Mazer, 1983; Shaklai *et al.*, 1984), which is elevated in human diabetics (Dolhofer and Wieland, 1980). The incubation of HSA with glucose results in its non-enzymatic glycoxidation in a concentration, incubation period and temperature dependant manner (Bayness *et al.*, 1984). The principal site of glycation of HSA is lys-525, but the lysine residues in positions 199, 281 and 439 are also sussceptable to glycation. In addition there are six more residues that glycate less efficiently (Shaklai *et al.*, 1984).

The *in vitro* exposure of protein to glucose results in the non-enzymatic covalent attachment of glucose to lysine side chains in a manner that observed *in vivo* (Brownlee *et al.*, 1987). This process also occurs in individuals with normal control of plasma glucose concentrations, but HSA is typically three times more glycated than the rest of the population in conditions of hyperglycemia (Bourdon *et al.*, 1999) where, during periods of poor control of plasma glucose concentration, circulating levels of 25 mM glucose have been recorded. The interaction of HSA with glucose showed two sets of binding site. The first set

consists of two sites with cooperativity and the second set consists of nine identical non-cooperative sited (Mohamadi-Nejad *et al.*, 2002). The non-enzymatic glycation of HSA induces refolding of globular proteins, accompanied by formation of cross-β structure that may leads glycated albumin into fibrous or amorphous aggregates (Bouma, *et al.*, 2003) which may change its secondary and tertiary structure (Coussons, *et al.*, 1997), alter its normal functions (Shaklai *et al.*, 1984; Iberg and Fluckiger, 1986). Compared to the original albumin, AGE molecules are not only larger in size but also have lower isoelectric points and carry more negative charges. Both the size and the negative charges of AGEs continue to increase over time during incubation (Wu, *et al.*, 1996) and this may also changes the recognition of proteins (Bitensky *et al.*, 1989). Abnormalities in protein recognition may contribute to the pathological impact of glycation (Bitensky *et al.*, 1989).

Free radical biochemistry

It is believed that life originated as a result of free redical reactions (FRRs), selected FRRs play major metabolic roles, causing repeated mutation, and death, thereby assuring evolution. Further, life span evolved in parallel with the ability of organisms to cope with damaging free radical reactions. In short, the origin and evolution of life may be due to free radical reactions and, in particular, to their ability to induce random change (Harman, 2001).

Oxygen free radicals are believed to be generated by a number of processes *in vivo*, including the 'respiratory brust' of phagocytic cells, metal catalyzed substrate autoxidations, mitrochondrial electron transfer and the reduction of hydroperoxides (Halliwell and Gutteridge, 1984).

Regardless of how and where free radicals are generated (exogenously or intracellular), a rise in the oxidant level has two important effects: damage to various cell components and triggering the activation of specific signaling pathways NF-κB, ERK, JNK, NAPK, and PKC isoforms (Idris, *et al.*, 2001). Reactive oxygen species (ROS) generated during various metabolic and biochemical reactions having multifunctional effects that include oxidative damage to DNA leading to various human degeneration and autoimmune

diseases (Hasan *et al.*, 2003), moreover, also responsible for the elimination of invading pathogens (Khan *et al.*, 2005)

ROS encompasses a variety of diverse chemical species including superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), alkoxyl ($RO^{\cdot}$), peroxyl ($ROO^{\cdot}$), hydroxyl radicals ($^{\cdot}OH$), and hydrochlorous acid (HOCl) (Jackus, 2000). ROS are highly reactive and have extremely short half-lives. Most estimates suggest that the majority of intracellular ROS production is derived from the mitochondria. There are numerous mechanisms for generation of ROS *in vivo* (Emerit *et al.*, 1990).

Superoxide anion radical

The uptake of one electron by molecular oxygen result in the formation of the superoxide anion radical ($O_2^{\bullet-}$) (Florence, 1990; Harris, 1992). Superoxide radical is a by-product of many of detoxification reactions (Beckman and Ames, 1998). First, superoxide is a strong base and can therefore abstract protons from a variety of the compounds. Second, $O_2^{\bullet-}$ is a potent reducing agent; it can reduce quinines reversibly to semiquinones and transition metal ions such as Fe^{3+} and Cu^{2+} into their reduced forms (Fe^{2+} and Cu^{+}). Third the superoxide ion is a nucleophile and may thus readily interact with a number of electrophilic agents. System generating $O_2^{\bullet-}$ has been observed to kill bacteria, inactivate viruses, damage enzymes and membrane and destroy animal cell culture (Fridovich, 1978; Halliwell, 1981).

The superoxide radical is generated within aerobic biological systems during both enzymatic and non-enzymatic oxidation. It is eliminated by conversion to H_2O_2 and O_2 by superoxide dismutase (Fridovich, 1983; 1986). The finding that $O_2^{\bullet-}$ is produced by some enzymes and is efficiently scavenged by others (McCord and Fridovich, 1968; 1969) led to the view that $O_2^{\bullet-}$ is an agent of oxygen toxicity. In this view the superoxide dismutases (SODs), which catalytically scavenge $O_2^{\bullet-}$, serve a defensive role (McCord *et al.*, 1971).

Hydrogen peroxide and the hydroxyl radical

Hydrogen peroxide is ubiquitous in the biological systems, formed by the divalent reduction of dioxygen or by dismutation.

During the stepwise one-electron reduction of molecular oxygen, hydrogen peroxide is the second intermediate. It may however also be generated directly via a two-electron reduction of molecular oxygen. Hydrogen peroxide is a stable molecule that is generated as an end product of a variety of oxidative reactions in living cells. It may, infact, act as both as oxidizing and as a reducing agents. In presence of transition metals, H_2O_2 is thought to give rise to peroxyl radicals and extreme reactive hydroxyl radicals via the Fenton reaction (O' Brein, 1969)

$$H_2O_2 + Fe^{2+}/Cu^{+} \longrightarrow Fe^{3+}/Cu^{2+} + {}^{\cdot}OH + OH^{-}$$

Or through reaction with a multiplicity of other agents (eg. the air pollutants NO or NO_2)

$$NO + H_2O_2 \longrightarrow {}^{\cdot}OH + HONO$$

$$NO_2 + H_2O_2 \longrightarrow {}^{\cdot}OH + HONO_2$$

The hydroxyl radicals are a highly reactive oxidizing agent that can react with a wide variety of organic molecules. It can abstract a hydrogen atom from essentially any hydrogen–carbon bond and under go addition reaction with aromatic systems at a reaction rate close to diffusion limit.

Photolysis is another important way to produce hydroxyl radical. UV irradiation is responsible for this reaction that can directly split H_2O_2 into two OH (Masaki *et al*, 1995; Mazellier *et al.*, 2004).

$$H_2O_2 \xrightarrow{h\nu} {}^{\cdot}OH + {}^{\cdot}OH$$

Singlet molecular oxygen

Another radical derived from oxygen is singlet oxygen, designated as $^1O_2$. This is an excited form of oxygen in which one of the electrons jumps to a superior orbital following absorption of energy. Ever since its discovery (Kantsky and deBruijn, 1931), its production by photosensitization reactions (including those involving endogenous sensitizer) has been intensively investigated but more recently attention has been drawn by studies showing that $^1O_2$ can also be generated in the absence of light for example by a number of enzymatic reaction (Gille and Joenje, 1991) or by interaction between superoxide and reduced glutathione (Wefers and Sies, 1983). Singlet oxygen is also produced during photoxidation of variety of biological compounds and xenobiotics (Krinsky, 1977; Krasnovsky, 1991). It has been established that human leukocytes can generates $^1O_2$ (Kanofsky *et al.*, 1988). $^1O_2$ is relatively long lived, with half time in the range of 4-50 μs, so that the diffusion of singlet oxygen is possible with in a radius of estimated to be in the range of hundred A° (Schnuriger and Bourdon, 1968; Moan, 1990). Reactions of singlet oxygen are physical and /or chemical (Kasha and Khan, 1970; Krasnovsky, 1979), as physical reactivity is characterized by photoemissive decay, while the chemical reaction are many fold, including, additions to olefins, forming dioxytanes, allylic hydroperoxide, ('ene reaction') and endoperoxide, as well as oxide of sulphide or phenol to form sulphoxide or hydroperoxidienones (Aubry, 1991). This chemical reactivity is the bases of biological damage inflicted by singlet oxygen.

Autoxidation and glycation

Glycation is a major source of ROS that is generated by oxidative pathways of glycation (Rahbar and Figarola, 2003). Several studies support the idea that glycation and oxidation are closely linked processes, glucose autoxidation plays an essential role in non-enzymatic glycation of protein.

In vitro glycated serum albumin showed possible interactions between glycation and oxidation (Traverso *et al.*, 1997). Contents of the glycated serum albumin are an index of oxidative modification during glycation (Fu *et al.*, 1992).

Glycation mediated modifications induced free radicals can cause conformational changes which alter its antioxidant properties (Bourdon *et al.*, 1999).

Glucose exists in equilibrium with their enediol, which can undergo autoxidation to form an enediol radical. This radical reduces molecular oxygen to generate the superoxide radical and becomes oxidized itself to a dicarbonyl ketoaldehyde that reacts with protein amino groups forming a ketoamine. Ketoamine are similar to, although more reactive, than Amadori products and participate in AGE formation (Ahmed, 2005).

In hyperglycemic conditions, most of the carbonyl compounds generated by glycation need oxidative steps in their formation. The protein dicarbonyl compounds can participate in AGE formation and referred to as glycoxidative products (Liggins and Furth, 1997).

Free radicals and glycation (also called glycosylation) are central to chronic diseases, degeneration and aging. One important source of free radicals is advanced glycation end products (AGEs) resulting from non-enzymatic glycation and oxidation of proteins and lipids (Thomas *et al.*, 2005).

There is ample evidence that ageing, and thus life span, correlate with free radical generation and antioxidative defense as well as with advanced glycation end products. Most chronic diseases are also associated with free radicals and AGEs (Harman, 1984). Overproduction of free radicals accelerates cell ageing and is counteracted by antioxidants. The analysis of the mechanism generating free radicals and of the reaction of AGEs with cellular metabolism opens new avenues for the delaying of the development of chronic diseases such as atherosclerosis and neurodegenerative disease (Giardino *et al.*, 1998).

For a number of age-associated diseases, there is evidence of an association with free radicals and AGEs. Table 1 lists these diseases (Stachelin, 1997).

Table 1: Age-associated diseases thought to be related to free radicals and AGEs

- Atherosclerosis
- Cancer
- Complications of diabetes
- Cataract

- Alzheimer's disease
- Parkinson's disease
- Amyotrophic lateral sclerosis

Diabetic individuals may exhibit elevated levels of iron and free copper ions (Cutler, 1978; Mateo *et al.*, 1978), which in the presence of glycated proteins *in vitro* have been shown to generate free radicals (Hunt, 1994). The accumulation of glycated material in tissues that contain free copper ion, contribute to the generation of free radical mediated damage. These highly reactive species are capable of causing oxidative degradation of protein *in vitro* (Hunt *et al.*, 1994; Smith *et al.*, 1992). The formation of α-dicarbonyl compounds is known to be an essential step for the cross-linking of proteins and subsequent free radical generation (Rahbar and Figarola, 2003). Methylglyoxal is increased 5-6 fold; in adult onset, non-insulin dependent diabetes mellitus, 2-3 fold. In the presence of oxidative stress, glycation of proteins by methylglyoxal is enhanced. This may underlie the link of glycation and oxidative stress with diabetic complications, and may also contribute to pathological processes of ageing (Harman, 1998).

Reactive oxygen species and protein oxidation

Organic free radicals and related eletrophilic metabolite can bind covalently to cellular proteins. Amino acids can undergo oxidative damage if they interact with oxygen free radicals. Even peptide bonds can be subject to oxidative modification by reactive oxygen species. The consequence of such oxidative protein modification, in general, is a loss of physiological function, as in the case of covalent binding of reactive organic metabolites to proteins (Bradford and Allen, 1997). As free radicals are capable of initiating chain reactions, cross-linking of soluble and/ or membrane-bound proteins, yielding larger aggregates. The direct consequence is a change in higher order structure of the protein (e.g., in the quaternary structure), which, in turn, results in a loss of biological function (Adams *et al.*, 1999; Schoonover, 2001; Dhalla *et al.*, 2000).

As another extracellular event that may mark protein for complete intracellular proteolysis, we have studied the action of autoxidizing sugars on

some soluble proteins. Partly consequent on radical generation, proteins are glycosylated. Protein fragmentation takes place, with BSA as target protein, using glucose and glyceraldehyde autoxidizing for one to eight days (Wolff and Dean, 1987).

Elevated radical flux lead to an increased rate of proteolysis of bulk of long half-life proteins in cultured cells (Vince and Dean, 1987) and of mitochondrially synthesized proteins (Dean and Pollak, 1985). A greater acceleration of intracellular protein degradation after radical attack can be observed in erythrocytes (Davis, 1986).

Thus protein damage by radicals is critical in many biological processes. This damage may lead to functional inactivation (Willson, 1983), but usually the inactivated proteins are degraded, so that proteolysis forms a secondary defence. However, when the target proteins are critical for rapid homeostatic mechanisms (as in the case of transport proteins) or when the proteolytic defence and/or other antioxidant defences are overwhelmed, toxic events may ensue.

Antioxidants

Antioxidants are key line of defence capable of scavenging free radicals by preventing radical formation, intercepting radicals from further activity (Cotgreave *et al.*, 1998). Antioxidant defences are primary and secondary. The defences that directly scavange, H_2O_2 and $^{\bullet}OH$ are known as primary antioxidant defence. Secondary antioxidant defences consist of the repair mechanisms that act on biomolecules that have undergone oxidative damage.

The major endogenous antioxidants are: 1) superoxide dismutase (SOD) which removes $O_2^{-\bullet}$, catalase that converts H_2O_2 to water (H_2O) and O_2, and glutathione peroxidase, which helps with H_2O_2 removal and prevents hydroxyl radical ($^{\bullet}OH$) formation (Fig. 3) (Halliwell and Gutteridge, 1999). Lipoic acid has the unique ability to regenerate several other antioxidants such as vitamin E, vitamin C (Packer and Coleman, 1999). Vitamin C, the major plasma antioxidant, is a scavenger of many ROS and reactive nitrogen species, and is capable of

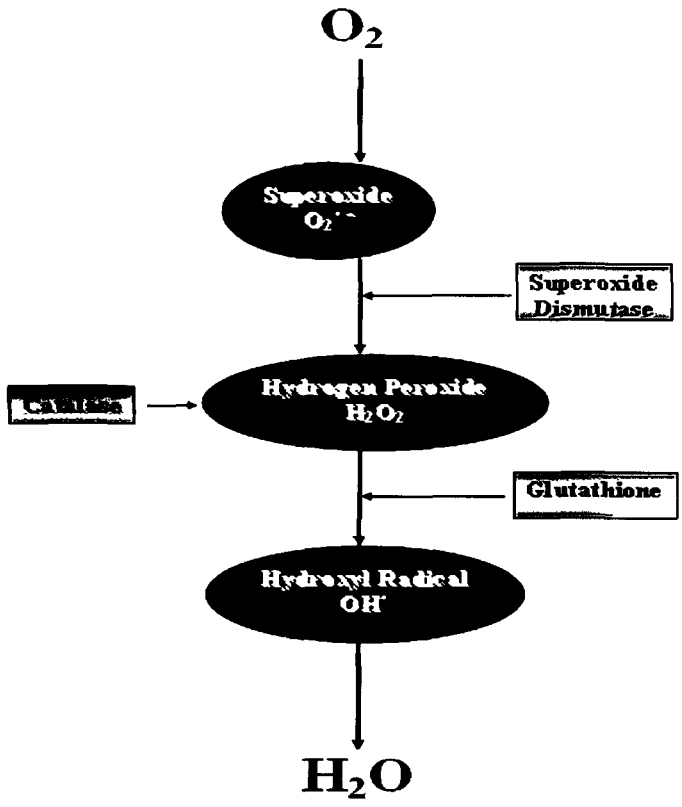


Fig. 3. Schematic representation of antioxidant activity of SOD, catalase and glutathione (Halliwell and Gutteridge, 1999).

regenerating tocopherol from its radical (Halliwell and Gutteridge, 1999; Maxwell, 1995). Metal chelators DETAPAC and EDTA sequester the trace amounts of transition metal and hence inhibiting oxidation (Wolff and Dean, 1987). Benzoic acid is also a hydroxyl radical scavenger, which inhibits protein fragmentation (Hunt *et al.*, 1988). There are numerous other dietary sources of antioxidants (McDermott, 2000).

Autoimmunity

The lack of an immune response to self when responses to environmental antigens are retained is due to immunological tolerance. The role of tolerance, or lack of tolerance, is important to the understanding of autoimmune diseases and transplantation immunobiology (Goldsby *et al.*, 2003). A loss of natural tolerance (to self) underlies all autoimmune diseases. Many more individuals develop autoimmune phenomena than autoimmune disease. Immune-mediated (Type I) diabetes results from an organ-specific autoimmune-mediated loss of insulin-secreting β cells. This chronic destruction process involves both cellular and hormonal components detectable in the peripheral blood, months or even years, before the onset of clinical diabetes (Kukreja and Maclaren, 1999).

In order to elicit an immune response, a molecule must be recognized as nonself by the biological system. When an antigen is introduced into an organism, the degree of immunogenecity depends on the degree of its foreignness, generally the greater the phylogenetic distance between two species, the greater the structural disparity between them. Bovine serum albumin is strongly immunogenic when injected into rabbit (Goldsby *et al.*, 2003)

Anti-HSA antibodies have been observed in diabetes (Eilat *et al.*, 1981), a five fold greater occurrence than in nondiabetic persons (Gregor *et al.*, 1986). Proteins containing AGEs are highly immunogenic and CML is one of the major epitopes recognised by anti-AGE antibodies (Reddy *et al.*, 1995; Ikeda *et al.*, 1996). The presence of AGE-antibodies in the serum of streptozotocin-diabetic rats as well as in a small number of diabetic patients have been reported (Shibayama *et al.*, 1999) AGE can exert their immunogenicity, demonstrate that presence of AGEs-immune complexes (AGE-IC) in the diabetic patients that may

play a role in the artherogenesis (Turk *et al.*, 2001). Interactions of AGE autoantibodies with AGEs as a continuously produced antigen result in the formation of AGE-ICs that may play role in diabetic complications (Jakus and Rietbrock, 2004). The analysis of the frequency distribution profile shows that 14% of the diabetic subjects display significant antibody binding to AGE-HSA than the control subjects (Vay *et al.*, 2000).

Diabetes mellitus

Diabetes is growing worldwide at an ever-increasing pace. The latest WHO estimate for the number of people with diabetes worldwide in the year 2000 was 177 million, which is expected to increase to 300 million by 2025. Around 4 million deaths per year are caused due to conditions directly or indirectly associated with diabetes.

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. In DM low insulin levels prevent cells from absorbing glucose. As a result, glucose builds up in the blood. When glucose-laden blood passes through the kidneys, all the excess glucose cannot be absorbed. This excess glucose spills into the urine, accompanied by water and electrolytes; ions required by cells to regulate the electric charge and flow of water molecules across the cell membrane. This causes frequent urination to get rid of the additional water drawn into the urine (polyurea); excessive thrust to trigger replacement of lost water (polydipsia); and hunger to replace the glucose lost in urination (polyphagia) (Alvin, 2005).

A wide spectrum of pathogenic processes is involved in the development of diabetes mellitus, which may range from autoimmune destruction of beta cells to the development of insulin resistance. The major risk factors of diabetes mellitus are age, obesity, lack of exercise, along with a strong genetic predisposition. A strong HLA association with some forms of the disease along with some specific antibodies has also been identified (Alvin, 2005).

Diabetes mellitus was broadly classified into two major types.

Type 1 diabetes mellitus

Type 1 or insulin dependent diabetes mellitus, also known as juvenile onset diabetes mellitus is characterized by an absolute deficiency of insulin where the patient is totally dependent on an exogenous source of human insulin. In over 90% of the cases Type 1 diabetes mellitus is immune mediated and in less than 10% of the cases it is idiopathic. It usually occurs in younger age group with highest incidence in 10-14 years age group. Improper glucose metabolism causes an increase in the blood levels of ketone bodies making the patient very prone to ketosis. The incidence of Type 1 diabetes shows little bias upto 15 years of age, but more males are diagnosed in early adult life. The lower prevalence of IAA in adolescent females implies sex specific modulation of the autoimmune process during puberty (Williams *et al.*, 2003).

Immune mediated diabetes results from a cellular mediated destruction of the β-cells of the islets of langerhans of the pancreas (Atkinson and McLaren. 1994). A number of antibodies have been detected as the markers of autoimmune diabetes. These include islet cell autoantibodies (ICA), insulin autoantibodies (IAA) (Atkinson *et al.*, 1992) GAD_{65} autoantibodies (Jun *et al.*, 2002), antibodies to tyrosine phosphatases IA-2 and IA-2*β* (Lan *et al.*, 1996; Lu *et al.*, 1996).

Even in the presence of the above predisposing factors the pathogenic process might not get initiated. Detailed studies have shown that a toxic or infectious insult is required for triggering the autoimmune destruction of β-cells of pancreas. Coxsackie-B virus, mumps virus and congenital rubella and cytomegalovirus have been shown to be related to Type 1 diabetes in this regard (Forrest *et al.*, 1971; King *et al.*, 1983; Karjalainen *et al.*, 1998; Pak *et al.*, 1988). Molecular mimicry has been closely linked to Type 1 diabetes mellitus in this respect where an autoimmune response is developed either against pancreatic antigen or against molecules of B cells resembling the viral protein.

Type II diabetes mellitus

Type II or non-insulin dependent diabetes mellitus, also known, as adult onset diabetes mellitus is the most common form of the diabetes mellitus. It is characterized by hyperglycemia secondary to insulin resistance and insulin levels that may be normal or even raised (Defronzo *et al.*, 1979; Turner et al., 1979; Kolterman *et al.*, 1981). A heterogenous group of factors is involved in its pathogenesis. Obesity has been a strong predisposing factor for the development of insulin resistance and hence Type 2 diabetes mellitus (Bogardus *et al.*, 1985; Olefsky *et al.*, 1982). Moreover, visceral obesity and not subcutaneous disposition of fat has been held responsible for giving rise to insulin resistance (Kissebah *et al.*, 1982). Lack of physical activity and increasing age has also been the precursors of this form of disease. Genetic predisposition is also a strong predisposing factor (Herman *et al.*, 1994; Bryne *et al.*, 1996).

Type II DM has a strong genetic component, major gene that predisposes yet to be identified, but it is clear that inheritance is polygenic and multifactorial. The concordance of Type II DM in identical twins is between 70 - 90%. Individual with a parent with Type II DM have raised risk of diabetes; if both parents with Type II DM, the risk is approximately 40%.

Environmental factors (examples, nutrition and physical activity) further modulate phenotypic expression of the disease. Genetic defect in insulin secretion or action may not manifest itself unless an environmental event of another genetic defect such as obesity is superimposed (Shervin, 2001; Alan, 1998).

Type II DM is characterized three pathophysiologic abnormalities:

1- Impaired insulin secretion

2- Peripheral insulin resistance

3- Excessive hepatic glucose production

In early stages β-cells compensate by increase in insulin production. So glucose tolerance remains normal despite insulin resistance. Insulin resistance and compensatory hyperinsulinemia progresses, the β-cells in certain individuals are unable to sustain the hyperinsulinemic state. A further decline in insulin secretion and an increase in hepatic glucose production leads to overt diabetes is

a predominant feature and results from combination of genetic susceptibility and obesity.

The pathogenesis of insulin resistance is currently focused on PI-3 kinase signaling defect, which reduces translocation of GLUT 4 to the plasma membrane. Another emerging theory proposes that increased levels of free fatty acids, a common feature of obesity, may contribute to pathogenesis. Free fatty acids can impair glucose utilization in skeletal muscles, promote glucose production by the liver and impair β-cell function.

The diabetes preventive programme demonstrated that intensive changes in lifestyle (diet and exercise for 30 min/day five times in a week) in individual with IGT prevented or delayed the development of Type II diabetes by 58 percent (Alvin, 2005).

Albumin in diabetes mellitus

In fact, only a small number of factors are known to result in the variation in serum albumin. The alteration in the structure of albumin due to uncontrolled hyperglycemia causes vascular complication (Bourdon *et al.*, 1999). Its level decreases about 30-40% in diabetes mellitus (Woodside *et al.*, 1998). Moreover, in diabetes the detection of an increase in albumin urinary excretion is a marker of some disease and disfunction of kidney (Keen and Chiouverkis, 1963: Howthorne, 1989). Alteration of redox state of HSA in patients with diabetes mellitus may be the important parameter for the pathogenesis of the disease, as oxidized form increased in diabetic patients (Suzuki *et al.*, 1992: Oettl *et al.*, 2005). Glycated HSA increases in the diabetic sera which is transportated into renal glomerulus, induce an increase in Type IV collagen production and a decrease in proliferate capacity by mesangial cells (Wolff *et al.*, 1991).

Advanced glycation end-products in the pathogenesis of diabetic complications

In summary, AGE products are important mediators of diabetic secondary complications and age-related diseases.

Diabetic retinopathy

It is characterized by abnormal vessel development leading to haemorrhages, ischaemia and infections. Specific morphological and functional changes include basement membrane thickening, loss of pericytes and increased permeability (Chappey *et al.*, 1997). Accumulation of AGE might contribute to this state of vasculopathy by increasing retinal endothelial cell permeability resulting in vascular leakage (Vlassara *et al.*, 1994).

A study comparing post-mortem human retinas between diabetic subjects with background and prolifirative retinopathy and non-diabetic retinas (Murata *et al.*, 1997), none of the control subjects showed CML immunoreactivity. *In vitro* studies have showed that AGE modification of retinal basement membrane results in reduced retinal pericyte proliferation but increased retinal endothelial cell proliferation. These effects are observed in diabetes and with AGE effects on nitric oxide depletion, reactive oxygen production and changes in vessel elasticity, could explain AGE effects on microvascular structure and function (Brownlee, 2000).

Diabetic nephropathy

Persistent hyperglycemia has a central role in the development of diabetic nephropathy that is clinically manifested by proteinuria progressing to renal insufficiency, and histopathologically by mesangial expansion and glomerular basement membrane thickening. A possible link between elevated glucose level and diabetic nephropathy resides in the glycation process producing AGEs. This modification can impair the original function of either protein and may affect normal processes of turnover and clearance (Monnier *et al.*, 1992; Berg *et al.*, 1997).

Circulating serum AGE level is markedly increased in patients with diabetes and renal insufficiency. Serum AGEs include both serum proteins that have been modified by advanced glycation and low molecular weight AGE peptides (Makita *et al.*, 1994). Using specific immunoassay, serum AGE peptide levels have been found to correlate with renal function. In normal controls, AGE peptide clearance has been estimated to be 0.72 ml/min. Diabetic persons with

normal glomerular filtration rate can clear AGE peptides at the same rate. However, progressive loss of renal function is associated with increasing circulating AGE peptide levels. In these patients, AGE peptides persist at up to 8-fold normal level (Turk, 2001).

Diabetic atherosclerosis

It has also been well documented that lipoproteins are deeply involved in the atherogenic process. Diabetes can lead to several lipoprotein modifications that can affect their interaction with arterial wall cells, thereby contributing to the increased risk of atherosclerosis. Glycation of apolipoproteins in all classes of circulating lipoproteins has been found in diabetes. Approximately 2% to 5% of apo B in the plasma of diabetic persons are glycated, compared with about 1% in the plasma from nondiabetic control subjects (Turk, 2001). Reactive AGE peptides have been experimentally shown to rapidly promote AGE-LDL formation *in vitro*, indicating that glucose may not be the most proximal reactant capable of producing AGE-LDL *in vivo*. AGE lipoproteins like other advanced glycation modified proteins, bind to specific receptors on macrophages and other cell types, and can stimulate the release of cytokines and growth factors which may play a role in atherogenesis (Lopes-Virella *et al.*, 1988; Vlassara, 1996). Thus, a reduction in the level of glycation of lipoproteins as well as of the arterial wall extracellular matrix might alter the interaction of lipoproteins with the matrix and reduce their retention in the arterial wall where they are able to exert their atherogenic damage.

ROS in diabetes mellitus

The putative role of ROS in the development of diabetic complications has been investigated for several decades (Baynes and Thorpe, 1999; Oberley, 1988; Ceriello *et al.*, 2000). The level of ROS is increased in hyperglycemic condition, which may be closely associated with diabetic complications (Palm *et al.*, 2003). It is also described that the increased release of superoxide anions ($O_2^{\bullet -}$) from the uterine arteries of diabetic patients due to enhanced $O_2^{\bullet -}$ production in smooth muscle cell than the endothelial cells. The overproduction of ROS lowered

antioxidant defense and alterations of enzymatic pathways in humans with poorly controlled diabetes mellitus (Jakus, 2000).

Rheumatoid arthritis

Rheumatoid arthritis (RA) is inflammation of the joints along with the production of a number of autoantibodies (Newkrik *et al.*, 2003). In many part of the world the disease is called rheumatism. It is named after the town where it first appeared. It can be caused by bacteria, virus, by a disruption of body chemistry or by high stress. A predominant feature of synovitis in rheumatoid arthritis (RA) is the development of a hypertrophic, oedematous and highly vascularized so-called pannus-like tissue that progressively invades and degrades adjacent cartilage and bone (Gay *et al.*, 1993; Firestein, 1996; Pap *et al.*, 2000). This tissue, which originates from the synovial lining layer of affected joints, consists of synovial fibroblasts (SFs), synovial macrophages and various infiltrating inflammatory cells such as activated T and B lymphocytes. The joint destruction is mediated by matrix-degrading enzymes released predominantly by activated SFs similar to mechanisms observed in malignant disease (Muller-Ladner *et al.*, 1998).

Long term outcome predictors, such as the presence of rheumatoid factors (Eberhardt *et al.*, 1990; Van Schaardenburg *et al.*, 1993) and the QKRAA shared epitopeon the HLA-DRβ chain (Wagner *et al.*, 1997; Weyand *et al.*, 1992).

Albumin in rheumatoid arthritis

Albumin is the largest transportable source of nitrogen and energy in the body and can be used to carry drugs to cells with a high nitrogen and energy demand such as the cells involved in the synovial inflammation of RA (Wunder *et al.*, 2003). Recently shown that methotrexate-HSA (MTX-HSA) is more effective than equivalent concentrations of MTX in preventing CIA (Wunder *et al.*, 2003). In addition to synovial fibroblasts, which we previously identified as target cells for albumin-mediated drug delivery in RA, monocytes, granulocytes and T and B lymphocytes, which all were shown to play a significant role in the

pathophysiology of this disease, might be further targets of this novel treatment approach. (Fiehn *et al.*, 2004). Albumin conjugates are internalized into cells by endocytosis.

The potential use of albumin as a drug carrier in RA is favoured by the facts that the permeability of the blood–joint barrier for albumin in inflamed joints is markedly increased and patients with active RA, similar to cachectic cancer patients. A complex of human serum albumin and rabbit IgG anti-HSA antibodies is used as antigen for RF (Kleveland *et al.*, 1988). Moreover, MTX-HSA is superior to MTX in the suppression of tumour growth and development of arthritis in rodent models (Fiehn *et al.*, 2004).

Glycation and rheumatoid arthritis

Rheumatoid arthritis (RA) is characterized by persistent articular and systemic inflammation, along with the production of a number of autoantibodies. Antibodies directed toward the Fc portion of the IgG molecule or rheumatoid factor (RF) are detected in approximately 70% of patients with RA.

During inflammation proteins can be damaged by non-enzymatic glycoxidation (Singh *et al.*, 2001; Ulrich and Cerami, 2001). AGE-damaged proteins are increasingly being implicated in other diseases such as atherosclerosis, amyloidosis, aging (in particular, cartilage and the lens of the eye) (Ulrich and Cerami, 2001), also in RA (Chen *et al.*, 1998; Furumitsu *et al.*, 2000; Miyata *et al.*, 1998) and osteoarthritis (Torchiana *et al.*, 1998; Pokharna *et al.*, 1995). The cross-links that form in cartilage due to pentosidine, which cause the typical yellowing (Monnier *et al.*, 1984), are thought to contribute directly to the joint pathology and increase in urinary AGE detected in patients with osteoarthritis or RA. Such increases may also reflect cartilage degradation.

Not only cartilage but also antibodies can be damaged during inflammation. Previous studies have shown that AGE-damaged IgG can be detected in patients with arthritis of long duration (Ligier *et al.*, 1998; Lucey *et al.*, 2000; Newkirk *et al.*, 1998). AGE can be detected on both the heavy and light chains of IgG (Ligier *et al.*, 1998; Tai and Newkrik, 2000; Lapolla *et al.*, 2000). Studies indicated not only that IgG-AGE could be detected in patients with RA, but also that

approximately 30–40% of RF-positive patients mounted an immune response to IgG-AGE. One possible explanation for the origins of these antibodies and the link to RFs is that RF-positive B-cells could act as antigen-presenting cells for the damaged IgG and thus stimulate the anti-IgG-AGE response (analogous to epitope spreading) by other antigen-selected B-cells that express a surface immunoglobulin specific for the IgG-AGE. In a previous study of RA patients with long standing disease (Lucey *et al.*, 2000), the anti-IgG-AGE antibodies were found to correlate significantly with measures of disease activity whereas RFs did not.

Patients with early disease, anti-IgG-AGE was found to correlate significantly with the swollen joint count and thus appears to be a marker of disease activity.

ROS in Rheumatoid arthritis

Recent studies indicated that increased oxidative stress and/or defective antioxidant status contribute to the pathology of rheumatoid arthritis (RA) (Karatas *et al.*, 2003). The study showed raised levels of malondialdehyde and low levels of endogenous antioxidants in patients of rheumatoid arthritis. Plasma catalase had also been reported to be significantly lower in patients with RA (Kamanli *et al.*, 2004). Another study reported impaired glutathione reductase activity in synovial fluid in rheumatoid arthritis (Bazzichi *et al.*, 2002). In active R (De Leo *et al.*, 2002) and juvenile idiopathic arthritis (Gotia *et al.*, 2001), increased oxidative stress and decreased levels of antioxidants have been reported. An epidemiological study (Knekt *et al.*, 2002) suggested that low selenium status may be a risk factor for rheumatoid factor-negative RA and low α-tocopherol status may be a risk factor for RA independently of rheumatoid factor status. Another study (Kerimova *et al.*, 2002) suggested that ROS generation could be decreased via inhibition of an enzyme (thioredoxin reductase) by gold thioglucose in RA. Overall it is well evident that there is increased state of oxidative stress in RA, which proposes the use of antioxidant supplementation in such patients.

Objective of the present study

Advance glycation end products produced by covalent interaction between sugar and free amino groups of proteins along with free radical production are known to be involved in the pathogenesis of several diseases like diabetes and rheumatoid arthritis. These free radicals and glycation end products are known to cause severe protein damage resulting in major structural alterations, which is implicated either through their functional disability or these being highly immunogenic.

Thus based on earlier observations the present study was planned to study the effect of glucose and oxygen reactive oxygen species ($^{\cdot}OH$) on HSA, as it being most abundant protein in human blood. It was first modified with glucose and the glycated HSA was then subjected to hydroxyl radical ($^{\cdot}OH$), generated by UV irradiation in presence of hydrogen peroxide. Native and modified HSA were then characterized by various electrophoretic, spectral, circular dichroism spectropolarimetry, colorimetric and thermal denaturation studies.

Furthermore, antigenecity of native and modified HSA was probed by inducing antibodies in rabbits. Both native and modified HSA induced high titer antibodies. However, glycated HSA and ROS-glycated HSA were found to be more immunogenic in comparison to its native form, as assessed by direct binding ELISA. The specificity of induced antibodies was evaluated by competition ELISA and gel retardation assay.

The serum of patients with diabetes and rheumatoid arthritis contains a bewildering variety of autoantibodies that react with a variety of protein and nuclear antigens. In order to assess the possible role of glycated HSA and ROS-glycated HSA epitopes in the aetiology these diseases, sera from diabetic and rheumatoid arthritic patients were investigated for the presence of antibodies to native and modified HSA. The polyclonal anti-native and modified HSA antibodies could be used as probe to detect and quantitate protein modified with glucose and hydroxyl radical in healthy and disease.

More sensitive methods of quantitation of HSA modified with glucose and/or $^{\cdot}OH$ might serve as a better diagnostic and prognostic marker of disease process.

EXPERIMENTAL

MATERIALS

Human serum albumin (HSA), dinitrophenylhydrazine (DNPH), Millipore filter (0.2 μm), catalase, Protein A-Agarose (2.5 ml pre-packed column), mannitol, diethylene triaminepentaaceticacid (DETAPAC), ethylenetetradiaminetetraacetic acid (EDTA), aminoguanidine, Nitrobluetetrazolium (NBT), superoxide dismutase (SOD), bovine serum albumin (BSA), anti-human/anti-rabbit IgG-alkaline phosphatase conjugate, p-nitrophenyl phosphate, Coomassie Brilliant Blue G-250 and R-250, sodium dodecyl sulphate (SDS), Tween 20, Freund's complete and incomplete adjuvants, and agarose were from Sigma Chemical Company, U.S.A. D-glucose was obtained from Merck (India). 2,4,6-trinitrobenzenesulphonic acid (TNBS) were purchased from SRL Chemicals (India). Sephacryl^{TM}S–200 HR was purchased from Pharmacia Fine Chemicals, Sweden. Folin-Ciocalteau reagent and Blue Dextran 2000 were purchased from Center for Biochemical Technology, New Delhi. Absolute ethanol was obtained from BDH Laboratory Supplies, England. Polystyrene microtitre flat bottom ELISA plates having 96 wells (7 mm diameter) were purchased from NUNC, Denmark. Acrylamide, ammonium persulphate, bisacrylamide, N,N,N'N'-tetramethylethylenediamine (TEMED) were from Bio-Rad Laboratory U.S.A. Hydrogen peroxide, isoamyl alcohol, methanol, phenol and glacial acetic acid were from Qualigens, India. Diphenylamine was chemically pure. All other reagents/chemicals were of the highest analytical grade available.

Equipments

Shimadzu UV-240 spectrophotometer equipped with thermoprogrammer and controller, JASCO J-810 spectropolarimeter, Hitachi F_{2000} spectrofluorometer (Japan), ELISA microplate reader (Labsystem Multiskan, Finland), Elico pH meter LI-120, Ultraviolet lamp (Vilber Lourmat, France), Avanti 30 table top high speed refrigerated centrifuge (Beckman, U.S.A.), polyacrylamide gel electrophoresis assembly (Bio-Rad, U.S.A.) were the major equipment used in this study.

Collection of sera and blood samples

Normal human sera were obtained from healthy subjects. Diabetes mellitus patients and rheumatoid arthritic patients' sera were obtained from outdoor and indoor patients of the Department of Medicine, Department of Microbiology and Department of Orthopadics, Jawahar Lal Nehru Medical College Hospital, A.M.U., Aligarh. Serum samples were decomplemented at 56°C for 30 min and stored in aliquots at -20°C with 1% sodium azide.

Methods

Protein estimation

Protein was estimated by the methods of Lowry *et al.*, (1951) and Bradford (1976).

Protein estimation by Folin's-phenol reagent

The protein estimation by this method utilizes alkali (to keep the pH high). Cu^{2+} ions (to chelate proteins) and tartarate (to keep the Cu^{2+} ions in solution at high pH).

(a) Folin-Ciocalteau reagent

The reagent was purchased from Centre for Biochemical Technology, New Delhi and diluted 1:4 with distilled water before use.

(b) Alkaline copper reagent

The components of alkali copper reagent were prepared as follows:

(i) 2 percent sodium carbonate in 100 mM sodium hydroxide

(ii) 0.5 percent copper sulphate in 1.0 percent sodium potassium tartarate.

The working reagent was prepared fresh before use by mixing components (i) and (ii) in the ratio of 50:1.

(c) Procedure

To 1.0 ml of protein sample was added 5.0 ml of alkaline copper reagent and incubated for 10 min. at room temperature. 1.0 ml of working Folin-Ciocalteau reagent was added and the tubes were read at 660 nm after 30 min. The concentration of protein in unknown sample was determined from a standard plot of bovine serum albumin.

Protein estimation by Bradford (dye–binding) method

This assay is based on color change observed when Coomassie Brilliant Blue G–250, an acidic dye, binds hydrophobically to protein having positively charged groups (Bradford, 1976). In the environment of these positively charged groups, protonation is suppressed and a blue color is observed (λ_{max} =595 nm).

(a) Dye preparation

One hundred microgram of Coomassie Brilliant Blue G–250 was dissolved in 50 ml of 95% ethanol and was added 100 ml of 85% (v/v) orthophosphoric acid. The resulting solution was diluted to a final volume of 1.0 litre and filtered through a Whatman No.1 filter paper to remove undissolved particles.

(b) Protein assay

To 1.0 ml solutions, containing 10–100 μg protein, 5.0 ml of dye solution was added and the contents were mixed by vortexing. The absorbance was read at 595 nm after 5 min, against a blank reagent.

Modification of HSA by glucose

For the preparation of glycated HSA, 1mg/ml of HSA (15.15 μM), in 20 mM PBS, pH 7.4, with 0.05 M D-glucose filtered through 0.2 μm Millipore filter was incubated for 10 weeks at 37°C in dark under sterile conditions in capped vials. Solution of HSA without glucose served as control. After incubation, the

solutions were extensively dialyzed against PBS and stored at -20°C before use. Protein concentration was measured by Bradford method (Bradford, 1976).

IgG, BSA and poly-L lysine were also glycated similarly as mentioned above.

Modification of glycated HSA by reactive oxygen species

Aqueous solution of glycated HSA in PBS, pH 7.4 was irradiated under 254 nm light for 30 min at room temperature in the presence of hydrogen peroxide (10 mM). Under this condition the major radical generated would be 'OH. Excess of hydrogen peroxide was removed by extensive dialysis against PBS, pH 7.4.

Glycated IgG, BSA and poly-L lysine were also modified by ROS as mentioned above.

Polyacrylamide gel electrophoeresis

Electrophoresis was performed essentially according to the method of Laemmli (1970) using the slab gel apparatus manufactured by Biotech, India. Stock solution of 30% acrylamide containing 0.8% bisacrylamide was mixed in appropriate proportion to give the desired percentage of gel. It was then poured into the mould formed by two glass plates separated by 1.5 mm thick spacers. Bubbles and leaks were avoided. A comb providing a template for seven wells was quickly inserted into the gel film and polymerization allowed to occur. After 15-20 min, the comb was removed and the wells were cleaned, overlaid with running buffer. Samples containing 15-35 μg protein mixed with equal volume of sample buffer (containing 10% v/v glycerol, 0.06 M Tris HCl, pH 6.8 and traces of bromophenol blue as tracking dye) were applied to the wells. Eletrophoresis was performed at 50 V in the eletrophoresis buffer containing 0.025 M Tris and 0.2 M glycine until the tracking dye reached the bottom of the gel.

Sodium dodecyle sulphate polyacrylamide gel electrophoresis

Sodium dodecyle sulphate (SDS) was performed by the Tris-glycine buffer system of Laemmli (1970) using slab gel electrophoresis apparatus. Concentrated

stock solution of 30% acrylamide containing 0.8% bisacrylamide, 1.0 M Tris (pH 6.8 and 8.8) and 10% SDS were prepared and mixed in appropriate proportion to give the final required percentage. It was poured in the mould formed by two glass plates separated by 1.5 mm thick spacers avoiding leaks and bubbles. A comb providing a template for seven wells was quickly inserted into the gel before the polymerization began. The comb was removed once the polymerization was complete and wells were overlaid with the running buffer. Protein samples were prepared in the sample buffer containing 1% (w/v) SDS, 10% (v/v) glycerol, 0.0625 M Tris HCl, pH 6.8 and traces of bromophenol blue as tracking dye and where required 5% β-mercaptoethenol. The samples were boiled at 100°C for 5 min. electrophoresis was performed in electrophoresis buffer containing 0.025 M Tris and 0.2 M glycine at 50 V till the tracking dye reached the bottom of the gel.

Staining Procedures

After the electrophoresis was complete the gels were removed and the protein bands were visualized by staining.

(a) Coomassie brilliant blue staining

Protein bands were detected by staining with 0.1% coomassie brilliant blue R-250 in 40% Isopropanol and 10% acetic acid.

(b) Silver nitrate staining

The procedure described by Merril *et al.*, (1982) was followed. After electrophoresis the protein bands were fixed by rapidly immersing in a mixture of 40% (v/v) methanol and 10% (v/v) acetic acid for one hour with instant shaking. The gel was washed with 10% methanol and 5% (v/v) acetic acid twice, each time for 15 min to allow the gel to swell to normal size. This was followed by incubation in 3.4 mM potassium dichromate solution containing 3.2 mM nitric acid for 15 min and washing with distilled water. The washed gel was then immersed in silver nitrate solution for 20 min and then again washed with

distilled water and transferred to 280 mM solution of sodium carbonate containing 0.5% formaldehyde to make the gel alkaline. The reaction was stopped after 10 min by transferring the gel to 3% acetic acid solution for 5 min. The gel were washed 4 to 5 times with distilled water and finally stored in distilled water.

Spectral Analysis

Ultraviolet absorption spectroscopy

The ultraviolet absorption spectra of native and modified HSA samples were recorded in the wavelength range 200-400 nm on a Shimadzu UV–240 spectrophotometer. Using a cuvette of 1 cm pathlength. Two hundred fifty micrograms of native and modified HSA in a total volume of 3.0 ml was taken for spectral analysis.

Fluorescence spectroscopy

Native and modified HSA was analyzed by measuring intrinsic fluorescence at 25°C in Hitachi F_{200} spectrofluorometer (Japan). To study the cumulative effect of tyrosine, tryptophan and phenylalanine the protein was excited at 280 nm and the emission was measured over the range of 300-450 nm.

The fluorescence of tryptophan residue Trp_{214} in native and glycated HSA was monitored with excitation at 285 nm and the emission measured over the range 290-440 nm (Shaklai *et al.*, 1984). The concentration of protein samples was taken as 100 μM.

Circular dichroism spectropolarimetry

CD of native and modified HSA was measured in a Jasco J-810 spectropolarimeter equipped with a temperature controlled sample cell holder. Spectra were recorded with a scan speed of 20 nm/min and with a response time of 1 second. Each spectrum was the average of 2 scans. Measurements in the range of 200-350 nm were taken using HSA concentration of 2.2 μM with a 1 mm path length cell. For each sample the buffer was used as blank.

The results were expressed as mean residual electricity (MRE) and expressed in deg.cm^2.dmol^{-1}, which is defined as

MRE = θ_{obs}/ (10 x n x *l* x Cp)

Where θ_{obs} is the CD in milldegree, n is the number of amino acid residues, *l* is the path length of the cell and Cp is the mole fraction. The relative percentages of the secondary structure elements present were estimated by using software based on Yang equation (Chen and Yang, 1971).

Absorption-temperature scan

Thermal denaturation analysis of HSA was performed in order to ascertain the degree of modification incurred on the HSA by determining mid point melting temperature (Tm). Native and modified HSA samples were subjected to thermal denaturation on a Shimadzu UV-240 spectrophotometer coupled with a temperature programmer and controller assembly (Hasan and Ali, 1990). All the samples were melted from 30°C to 96°C at a rate of 1.0°C/min after 10 min equilibration at 30°C. The change in absorbance was recorded at 280 nm and percent denaturation evaluated with increase in temperature. Percent denaturation was calculated as follows:

$$\text{Percent denaturation} = \frac{A_1 - A_{30}}{A_{max} - A_{30}} \times 100$$

Where, A_T = Absorbance at a temperature T°C.

A_{max} = Final maximum absorbance on the completion of denaturation (96°C).

A_{30} = Initial absorbance at 30°C.

Ketoamine estimation by nitrobluetetrazolium

The native, glycated and ROS-glycated HSA were quantitated by a published colorimetric procedure (Mashiba *et al.*, 1992) using NBT with slight modification. BSA (10 mg/ml) was incubated with 0.5 M glucose for 90 days at 37°C in 20 mM PBS. The treatment resulted in complete modification of protein

with subsequent formation of ketoamines (Ahmed and Furth, 1991). Native, glycated and ROS-glycated HSA samples (50 µl) were added to the wells of 96 well microtitre plates in duplicate. One hundred µl of NBT reagent (250 µM in 0.1 M carbonate buffer, pH 10.35) was added to each well and incubated at 37°C for 2 h. The plate was read in a microplate reader at 550 nm. The amount of ketoamine in native, glycated and ROS-glycated HSA samples were calculated using standard curve constructed with glycated BSA.

Determination of protein bound carbonyl groups

HSA-bound carbonyl groups were estimated by a bublished procedure (Levine *et al.*, 1990). Briefly, 200 µl aliquot (containing 0.1 mg of protein) was mixed with 400 µl of 7 mM dinitrophenyl hydrazine (DNPH) in 2 M HCl. The mixtures were run in duplicate and the control protein samples were devoid of DNPH. After incubated for 1 hour at room temperature the DNP-hydrazones were precipitated by adding 500 µl of trichloroacetic acid (4% w/v) and centrifuged for 5 minutes at 14,000×g. The pellet was dispersed in ethanol-ethylacetate (1:1, v/v), in order to remove unreacted DNPH and centrifuged. After four such wash, the pellet was resuspended in 0.6 ml of 6 M guanidinium hydrochloride solution in 20 mM phosphate buffer, already adjusted to pH 2.3 with trifluoroacetic acid. The hydrazones were dissolved completely only by freezing overnight at -20°C and thawing. From the solution, 200 µl aliquot was taken into a microplate and read at 379 nm by a microplate reader. The results were expressed as the number of moles of carbonyl per mole of sample protein using a $\varepsilon_{379\ nm} = 22000\ M^{-1}cm^{-1}$.

Free amino group estimation

The exposed amino groups of native, glycated and ROS-glycated HSA were determined by the method described earlier (Habeeb, 1966) with slight modification. Suitable aliquots of the protein samples were dissolved in 1 ml of 0.1 M sodium tetraborate buffer, pH 9.3, was added 25 µl of 0.3 M TNBS and the tubes agitated instantly to ensure complete mixing and allowed to stand for 30

min at 25°C. Absorbance of the yellow color developed was recorded at 420 nm against a reagent blank. Glycine was used as standard amino acid.

Isolation of albumin from human serum

Serum albumin was isolated from normal and diabetic patient's blood according to the method described earlier (Tayyab and Qasim, 1990). Briefly, blood sample was centrifuged at 1500 x *g* for 20 min to obtain plasma, which was brought to 2.26 M in ammonium sulphate by adding requisite volume of 4.0 M ammonium sulphate solution, pH 7.0. The mixture was kept for 12 hr at room temperature and then carefully diluted with water and with frequent addition of 0.5 N H_2O_2, such that the final concentration of ammonium sulphate was reduced to 1.9 M and the pH to 4.2. After incubating it for 12 hr at room temperature, the precipitate was collected by centrifugation at 3000 x *g* for 30 min. It was washed 3 times with 2.2 M ammonium sulphate solution, pH 4.2 and then dissolved in 0.07 M sodium phosphate buffer, pH 7.4 containing 0.08 M NaCl. The protein preparation thus obtained was serum albumin, extensively dialysed against 6 litres of the 0.07 M sodium phosphate buffer pH 7.4 and stored at 4°C.

For further purification, 1.0 ml protein samples were applied to a gel filtration column (2×60 cm) packed with Sephacryl^{TM}S–200 (M.W. fractionation range 5×10^3-2.5×10^5 Dalton). After discarding the void volume (45 ml), determined by Blue Dextran, the serum albumin was eluted at a rate of one drop in 20 sec and thirty six fractions, each of 5 ml, were subsequently collected. Comparing the elution profile of commercially available unmodified HSA checked the purity of isolated albumin.

Isolation of IgG by Protein A-Agarose

Serum IgG was isolated by affinity chromatography on Protein A-Agarose column. Serum (0.5 ml), diluted with an equal volume of PBS, pH 7.4 was applied to a column equilibrated with the same buffer. The wash through was recycled 2–3 times. Unbound IgG was removed by extensive washing with PBS, pH 7.4. The bound IgG was eluted with 0.58% acetic acid in 0.85% sodium

chloride (Goding, 1976) and neutralized with 1 ml of 1.0 M Tris–HCl, pH 8.5. Three ml fractions were collected and read at 251 and 278 nm. The IgG concentration was determined considering 1.4 OD_{280} = 1.0 mg IgG/ml. The isolated IgG was dialyzed against PBS, pH 7.4 and stored at -20°C with 0.1% sodium azide.

Immunization Scheme

The animals (female rabbits) were immunized intramuscularly with 100 µg each of native and modified HSA, emulsified in Freund's complete adjuvant. Subsequent injections were in an incomplete adjuvant. Each animal received a total of 500 µg antigen during the course of immunization. Blood was collected from marginal vein of the ear. The serum separated from preimmune and immunized blood was decomplemented by heating at 56°C for 30 min. Pre-immune serum was collected prior to immunization. The sera were stored at -20°C in aliquots with sodium azide as preservative.

Immunological detection of antibodies

(a) Immunodiffusion

Immunodiffusion (ID) was carried out by Ouchtertony double immunodiffusion system (Ouchterlony, 1949). Six ml of 1.0% molten agarose in PBS, containing 0.1% sodium azide, was poured onto a glass petridish and allowed to solidify at room temperature. Wells of 5 mm diameter were cut into the hardened gel and an appropriate concentration of antigen and respective antiserum were placed in the wells. The petridish was allowed to stand in a moist chamber at room temperature for 48-72 hr. The gel was washed with 5% sodium citrate to remove non-specific precipitin lines and the result was analyzed visually.

(b) Enzyme–Linked Immunosorbent Assay

The following reagents were prepared in distilled water and used in ELISA.

Buffers and Reagents

→ Tris Buffered Saline (TBS)

- 10 mM Tris, 150 mM NaCl, pH 7.4.

→ Tris Buffered Saline–Tween 20 (TBS-T)

- 20 mM Tris, 144 mM NaCl, 2.68 mM KCl pH 7.4, containing 500 µl Tween 20.

→ Carbonate–Bicarbonate Buffer

- 15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6, containing 2 mM magnesium chloride.

→Substrate

- 500 µg/ml p-nitrophenyl phosphate in carbonate–bicarbonate buffer, pH 9.6.

Procedure

Antibodies were detected by ELISA using polystyrene microtitre plates as solid support (Ali and Alam, 2002).

One hundred microlitres of 2.5 µg/ml antigen in TBS, pH 7.4, was coated in test wells of microtitre plates, incubated for 2 hr at 37°C and then overnight at 4°C. The antigen-coated wells were washed three times with TBS–T to remove unbound antigen. Unoccupied sites were blocked with 150 µl of 1.5% BSA in TBS for 4-5 hr at room temperature. The plates were washed once with TBS-T and the antibody (100 µl/well) to be tested, diluted in TBS, was added to each well. After 2 hr incubation at 37°C and overnight at 4°C, the plates were washed four times with TBS-T and an appropriate anti-immunoglobulin alkaline phosphatase conjugate was added to each well. After incubation at 37°C for 2 hr,

the plates were washed four times with TBS-T, three times with distilled water and subsequently developed using p–nitrophenyl phosphate substrate. The absorbance was recorded at 410 nm on an automatic microplate reader. Each sample was run in duplicate. The control wells were treated similarly, but were devoid of antigen coating. Results were expressed as a mean of $A_{test} - A_{control}$

(c) Competition ELISA

The antigenic specificity of antibodies was determined by competition ELISA (Hasan *et al.*, 1991). Varying amounts of inhibitors (0-20 μg/ml) were mixed with a constant amount of antiserum or IgG. The mixture was incubated at room temperature for 2 hr and overnight at 4°C. The innate complex thus formed was coated in the wells. Percent inhibition was calculated using the formula:

$$\text{Percent Inhibition} = 1 - \frac{A_{inhibited}}{A_{uninhibited}} \times 100$$

(d) Band Shift Assay

For the visual detection of antigen-antibody binding and immune complex formation, gel retardation assay was performed (Dixit and Ali, 2004). A constant amount of antigen was incubated with varying amounts of IgG in PBS, pH 7.4 for 2 hr at 37°C and overnight at 4°C. One-tenth volume of 'stop-mix' dye was added to the mixture, which was electrophoresed on 5.5% polyacrylamide gel for 3 hr at 50 mV in SDS-PAGE buffer, pH 7.4. The gels were stained with silver staining and than the gel were kept in the distilled water.

RESULTS

Gel electrophoresis pattern of HSA

Polyacrylamide gel electrophoresis of native HSA, glycated HSA and ROS-glycated HSA was performed on 8% gel in the presence of β-mercaptoethanol (Fig. 4). Native HSA showed single band of about 65 kDa. After glycation of HSA and and its subsequent ROS modification, a visible difference in electrophoretic pattern was found. The glycated HSA showed broadening of band toward high and low molecular weight, more appreciably towards high molecular weight showing the formation of high and low molecular weight aggregates. ROS-glycated HSA showed decrease in the intensity and broadening of the band.

Spectroscopic analysis of native and modified HSA

Ultraviolet absorption spectral studies

The UV absorption spectra of glycated HSA showed hypochromicity at 280 nm. The hypochromicity was 43.5%, whereas, ROS-glycated HSA showed hyperchromicity at 280 nm to the extent of 32.3% (Fig. 5). However, no peak shift was found in both the cases.

Fluorescence spectroscopy

The glycated and ROS-glycated HSA samples were characterized for its fluorescence emission spectra over the range of 290–450 nm using excitation wavelength of 280 nm. As shown in Fig. 6, the emission spectra gave maximum intensity at 310 nm for native HSA, glycated HSA and ROS-glycated HSA. The glycated HSA showed decrease, whereas, ROS-glycated HSA showed increase in the magnitude of fluorescence intensity.

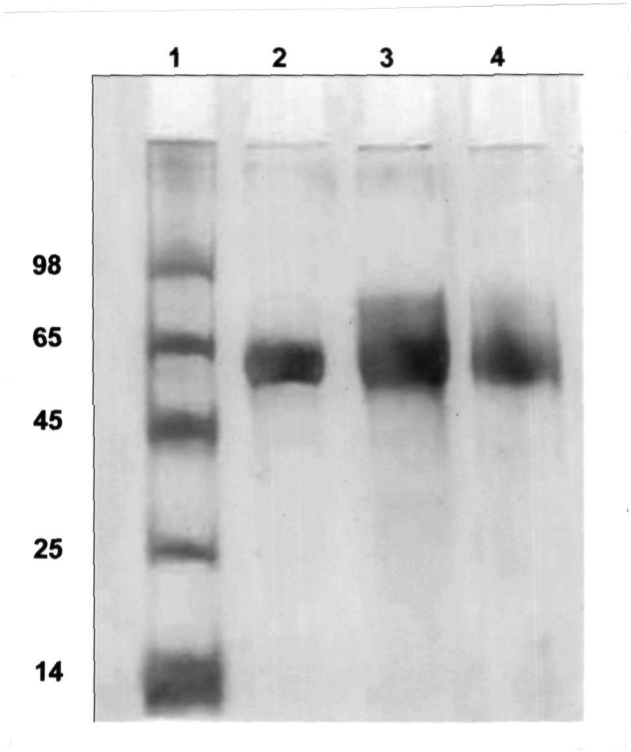


Fig. 4. SDS-polyacrylamide gel electrophoresis in presence of β-mercaptoethanol of native, glycated and ROS-glycated HSA. Sample proteins (10 μg) were loaded per lane in 10% polyacrylamide gel. Lane 1: protein markers (M_r, 98-14 kDa); lane 2: native HSA; lane 3: glycated HSA and lane 4: ROS-glycated HSA.

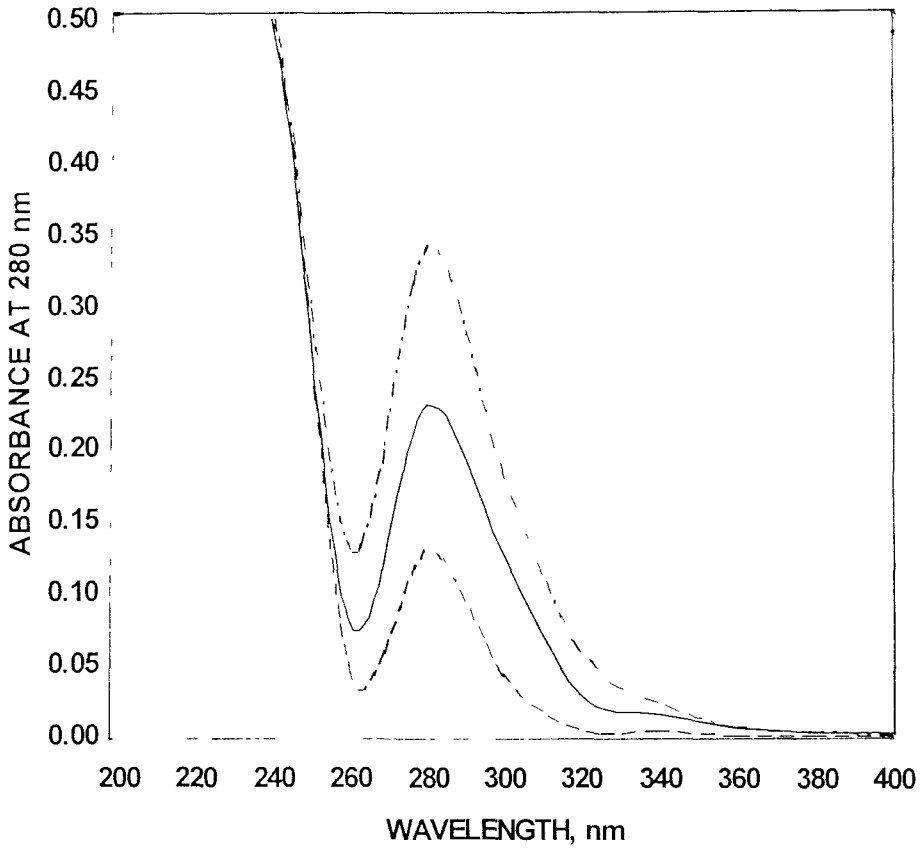


Fig. 5. Ultraviolet absorption spectra of native HSA (———), glycated HSA (— — —) and ROS-glycated HSA (· — · — ·).

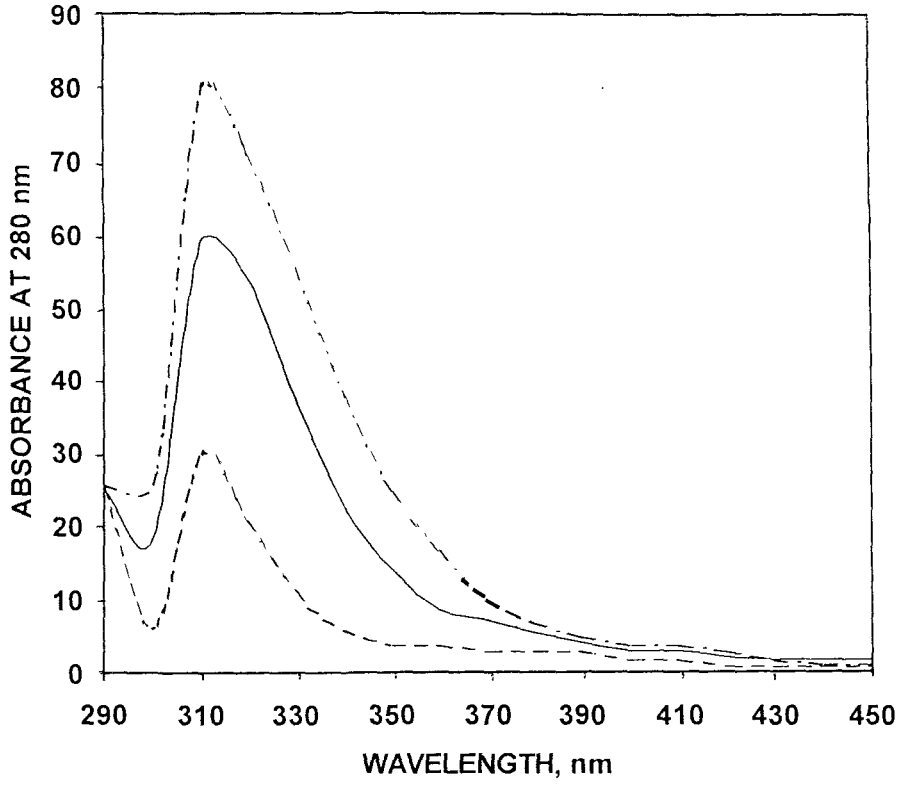


Fig. 6. Fluorescence emission spectra of native HSA (———), glycated HSA (– – –) and ROS-glycated HSA (– · – · – ·). Excitation wavelength was 280 nm.

Due to its high sensitivity and reproducibility, the level of fluorescence of a single tryptophan residue in the HSA molecule has been chosen as an index of conformational changes. Tryptophan specific fluorescence analysis were conducted, where both native as well as modified samples of HSA were excited at 295 nm. The emission maxima of native, glycated and ROS-glycated HSA samples were found to be 330, 310 and 321 nm, respectively, indicating a blue shift of 20 and 9 nm for glycated HSA and ROS-glycated HSA, respectively. There was marked reduction in the fluorescence intensity in glycated HSA, whereas, ROS-glycated HSA showed increased intensity as compared to native and glycated form of HSA (Fig. 7). The relative quantum yield of glycated HSA and ROS-glycated HSA, calculated from the areas of the emission peaks, were A_{GHSA}/A_{NGHSA}= 0.31 and A_{RGHSA}/A_{NGHSA}= 0.74 (where NGHSA, GHSA and R-GHSA represent native, glycated HSA and ROS-glycated HSA, respectively).

Circular dichroism spectropolrimetry of native and modified HSA

Induction of secondary structural changes on glycation and ROS-glycation can be revealed by the biophysical method of circular dichroic spectra. Changes in protein conformation were observed in UV C.D. spectra of native HSA, glycated HSA and ROS-glycated HSA (Fig. 8). In the spectral region of 200–250 nm, the CD signal of proteins is mainly due to its secondary structure (Hennessay *et al.*, 1981; Manavalan, 1993;). The CD spectra obtained for modified HSA and native HSA showed significant differences, indicating major changes in the secondary structure of both modified HSA samples compared to native HSA. The spectrum for both glycated and ROS-glycated HSA also exhibited a potent dip at 219 nm, inferring a stable β-sheet structure. The secondary structural details of native and modified HSA samples were obtained from software based on Yang equation (Chen and Yang, 1971) and are summarized in Table 1. Appreciable changes have been observed in α-helix, β-sheet, random coil and turns in HSA. Both modified HSA showed slight decrease in α-helix structure.

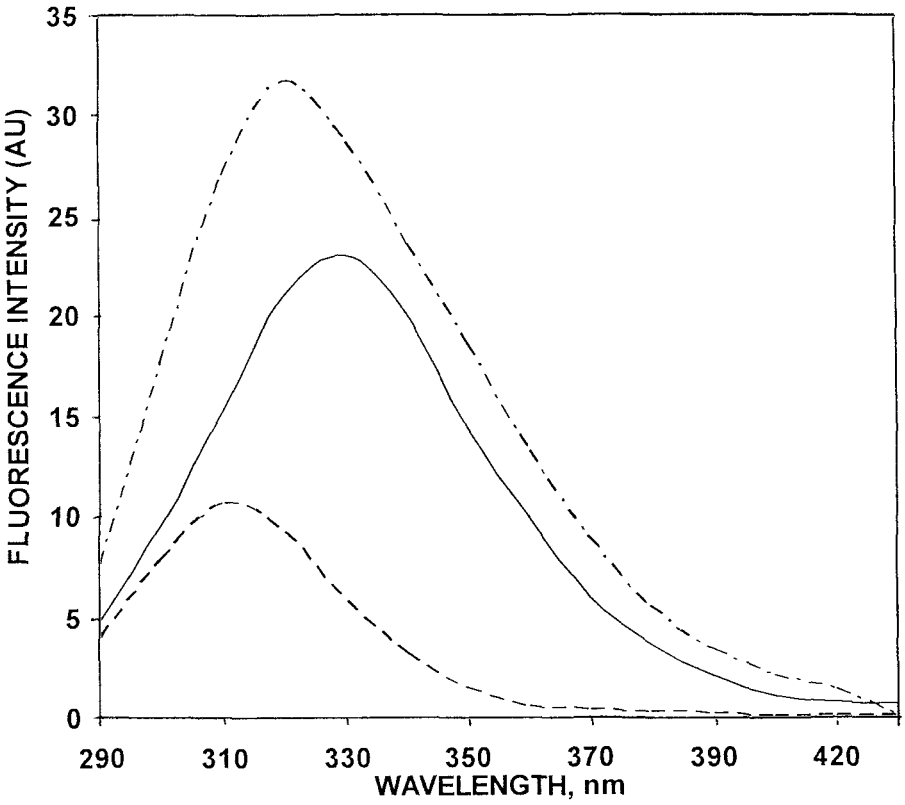


Fig. 7. Tryptophan fluorescence spectra of native HSA (———), glycated HSA (– – – –) and ROS-glycated HSA (– · – · – ·). Excitation wavelength was 295 nm.

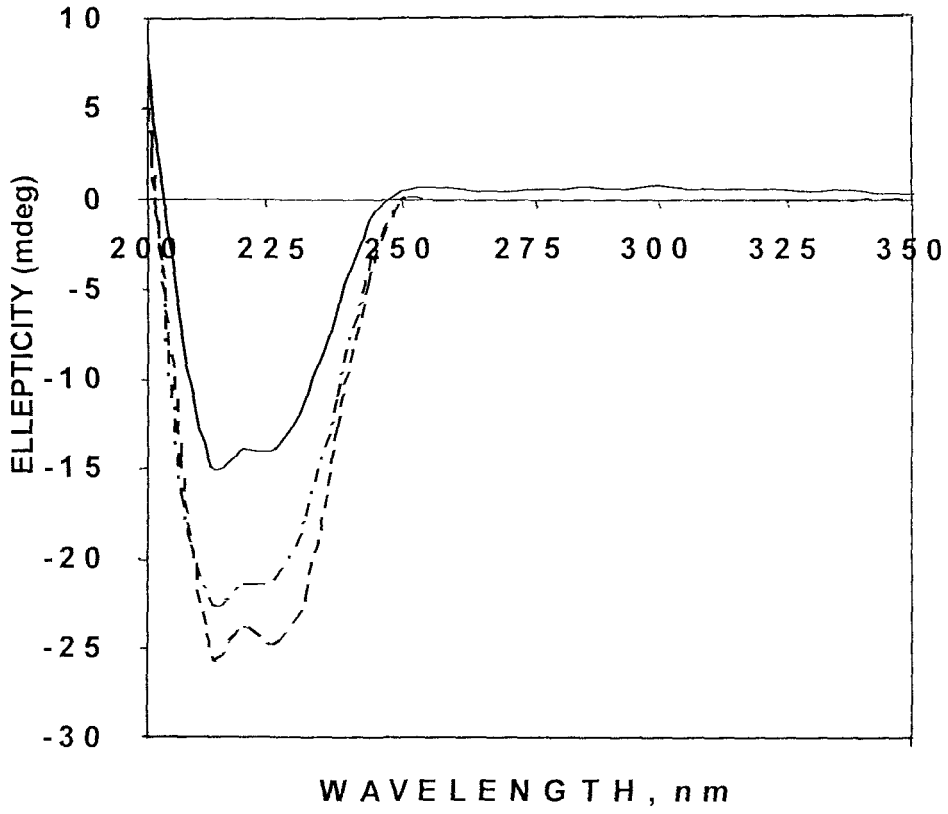


Fig. 8. Circular dichroic spectra of native HSA (———), glycated HSA (– – –) and ROS-glycated HSA (– · – · –).

Table 1

Secondary structure of native, glycated and ROS-glycated HSA observed by circular dichroism spectropolarimetry

Conformation	Native HSA	Glycated HSA	ROS-glycated HSA
α-helix	41.3 ± 1.08*	39.5 ± 0.86	38.01 ± 1.13
β-Sheet	28.4 ± 0.94	29.9 ± 0.79	28.83 ± 1.24
Random coil	11.7 ± 0.64	11.4 ± 0.71	12.01 ± 0.68
Turns	18.6 ± 0.76	19.2 ± 1.02	21.15 ± 0.89

* The values are in percent
Each sample was read in triplicates and the values are mean ± SD.

However, β-sheet showed increase after glycation and ROS modification. No appreciable changes were observed in the random coils for both modified HSA samples as compared to their unmodified form. However, the turns in the secondary structure of both modified samples of HSA showed gradual increase. These findings points out structural alterations in HSA and a partial destruction of the secondary structure of HSA on modifications.

Thermal denaturation of native and modified HSA

Thermally induced transitions were measured spectrophotometrically at 280 nm by heating protein samples at a rate of 1.5°C/min. Melting curves were obtained at temperatures from 30.0°C to 96.0°C. The increase in absorbance at 280 nm was taken as a measure of denaturation. The process was characterized by determining the percent HSA in denatured state as a function of temperature and by computing the melting temperature (Tm). Figure 9 shows thermal denaturation profile of native HSA, glycated HSA and ROS-glycated HSA. The melting temperature of native HSA at which 50% of the structural organization is lost was found to be 55.2°C, while in case of glycated HSA, it was found to be 74.3°C, a net increase of 19.1°C in the Tm value of glycated HSA was observed when compared to its unmodified HSA. Tm value for ROS-glycated HSA was found to be 64.0°C showing the net gain of 8.8°C and loss of 10.3°C compared with native HSA and glycated HSA, respectively.

Native HSA showed typical protein unfolding, evident after 40.0°C, however, for both glycated HSA and ROS-glycated HSA, the unfolding of the protein started at around 50.0°C, indicating thermal stability on glycation of native HSA and its ROS modification. Thermal denaturation characteristics of native and modified samples are summarized in Table 2.

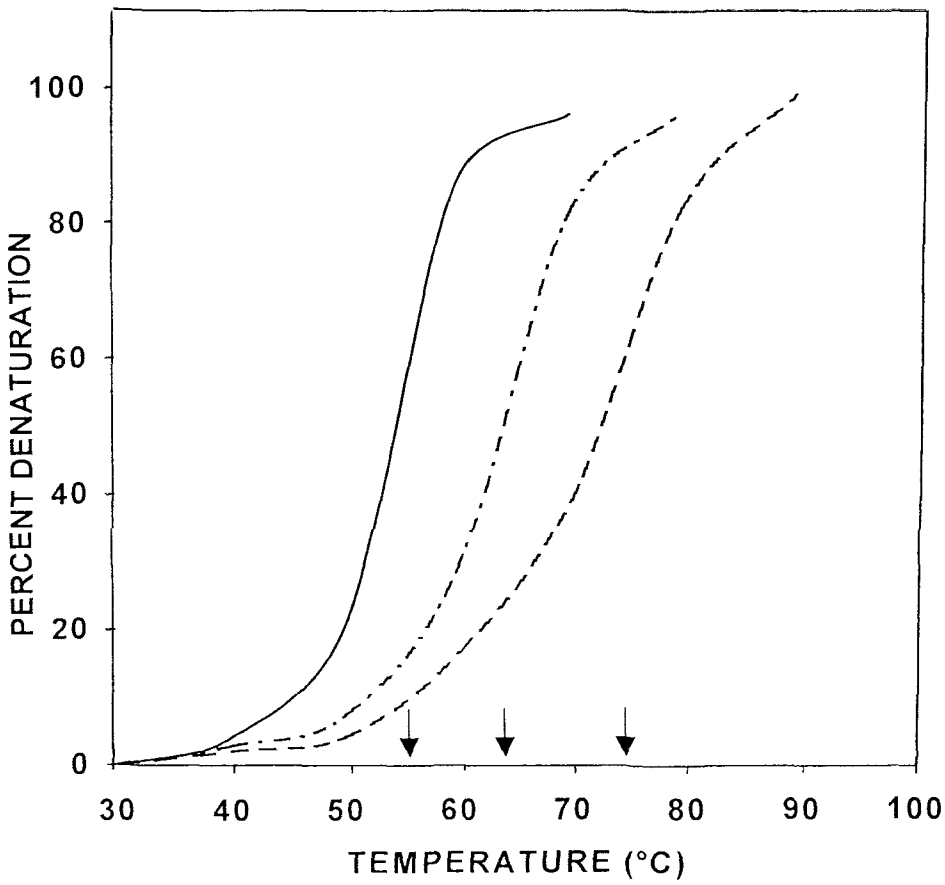


Fig. 9. Thermal denaturation profile of native HSA (———), glycated HSA (– – –) and ROS-glycated HSA (— • — • —).

TABLE 2

UV absorption and thermal denaturation characteristics of native, glycated and ROS-glycated HSA

Parameters	Native HSA	Glycated HSA	ROS-Glycated HSA
Absorbance at $A_{280\ nm}$	0.23	0.13	0.34
Percent chromicity at 95 °C		23.4 (hypo)	29.1 (hyper)
Melting temperature (Tm), °C	55.0	73.3	64.0
Onset of protein unfolding on melting (°C)	40.0	50.0	50.0

Ketoamine estimation of native and modified HSA

The ketoamine moieties formed by the glycation and ROS modification of albumin were measured calorimetrically by using NBT (Fig. 10). The formation of ketoamine was found to be 11.2 ± 0.6 and 8.3 ± 0.4 moles/mole of HSA for glycated and ROS-glycated HSA, respectively. The control non-glycated HSA gave a negligible ketoamine concentration of 0.3 ± 0.2.

Protein bound carbonyl groups

Carbonyl content bound to glycated and ROS-glycated HSA was estimated by reaction with DNPH. Significant amounts of carbonyl groups were produced on glycation and ROS modification of HSA. The carbonyl contents of glycated HSA and ROS-glycated HSA were detected to be 0.31 ± 0.06 and 0.44 ± 0.08 moles/mole HSA, respectively (Fig. 11). However, the native HSA showed negligible amount of protein bound carbonyl group, 0.05 ± .02 moles/mole HSA.

Determination of number of free amino groups

The number of free amino groups present in native HSA, glycated HSA and ROS-glycated HSA was evaluated (Fig. 12). There is decrease in free amino group contents with glycation of HSA, however, after ROS modification on the glycated HSA significant increase in the amino group as compared to native and glycated HSA. The number of free amino groups in glycated HSA and ROS-glycated HSA were found to be 7 ± 2 and 29 ± 4 amino groups per mol of HSA, respectively. The native HSA showed 16 ± 2 amino groups per mol of HSA.

Quenching studies

To examine the glycation of HSA and its oxidation by ROS has been affected by scavengers, metal chelators, and non-enzymatic and enzymatic antioxidants. Mannitol and sodium azide are scavengers of $^{\cdot}OH$ and $O_2^{\cdot-}$, respectively. Metal chelators (DETAPAC, EDTA), non-enzymatic antioxidant

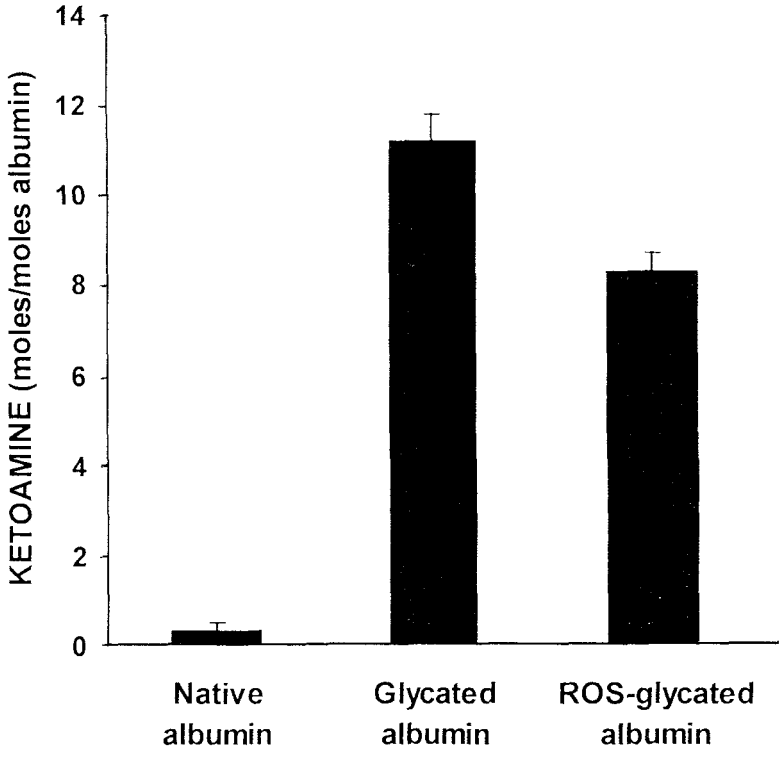


Fig. 10. Level of ketoamines in native HSA, glycated HSA and ROS-glycated HSA.

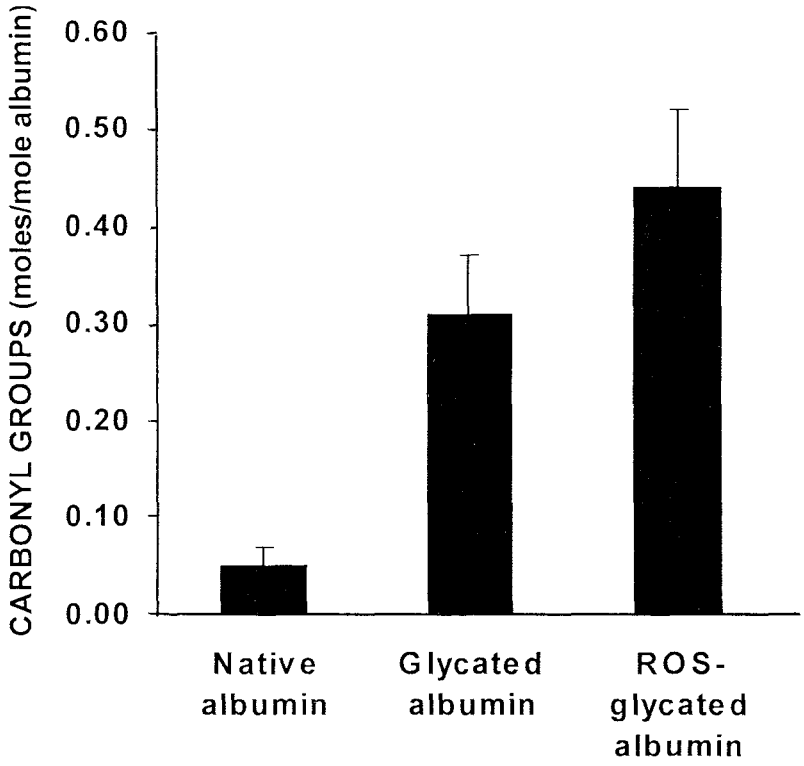


Fig. 11. HSA bound carbonyl groups in native HSA, glycated HSA and ROS-glycated HSA.

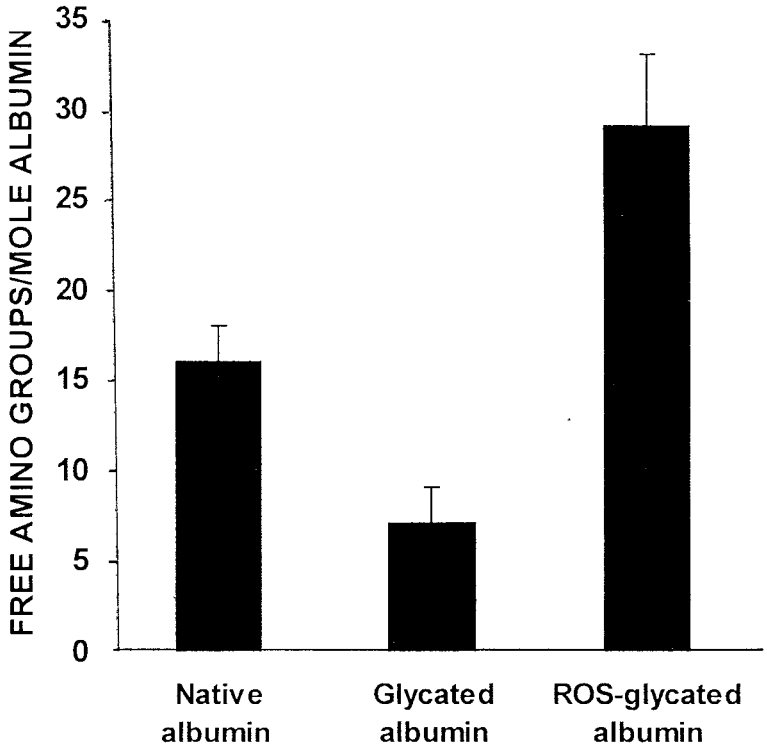
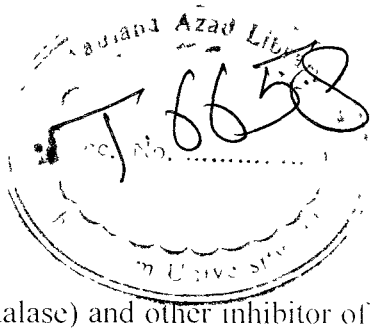


Fig. 12. Number of free amino groups exposed to the solvent system in native HSA, glycated HSA and ROS-glycated HSA.



(ascorbic acid), enzymatic antioxidants (SOD and catalase) and other inhibitor of glycation like aminoguanidine were also used.

Aminoguanidine caused maximum (~ 80%) inhibition of glycation (Fig. 13). Mannitol exhibited 34% inhibition. Ascorbic acid and sodium azide gave 30% and 25% inhibition, respectively. Enzymatic antioxidant SOD and catalase exhibited small decrease in glycation of HSA (25% and 10% inhibition). Metal chelators DETAPAC and EDTA showed remarkable inhibition (65% and 55%, respectively) in glycation of HSA.

As shown in Figure 14, combination of enzymatic antioxidant SOD and catalase exhibited maximum (~ 70%) inhibition of modification by reactive oxygen species, whereas, both SOD and catalase saparetly showed 40% and 52% inhibition. Mannitol exhibited significant (65%) inhibition. Ascorbic acid and sodium azide showed 40% and 25% inhibition, respectively, due to reactive oxygen species. Metal chelators DETAPAC and EDTA also showed significant inhibition in free radical modification (55% and 50%, respectively).

Immunogenicity of native and modified HSA

The Immunogenicity of native and modified HSA was determined by inducing antibodies in rabbits against native HSA, glycated HSA and ROS-glycated HSA. The antigenic specificity of induced antibodies were assayed by direct binding and comptetion ELISA. The antigen-antibody bindings were also assayed by immunodiffusion. The binding of these antibodies to the immunogens was further ascertained by band shift assay.

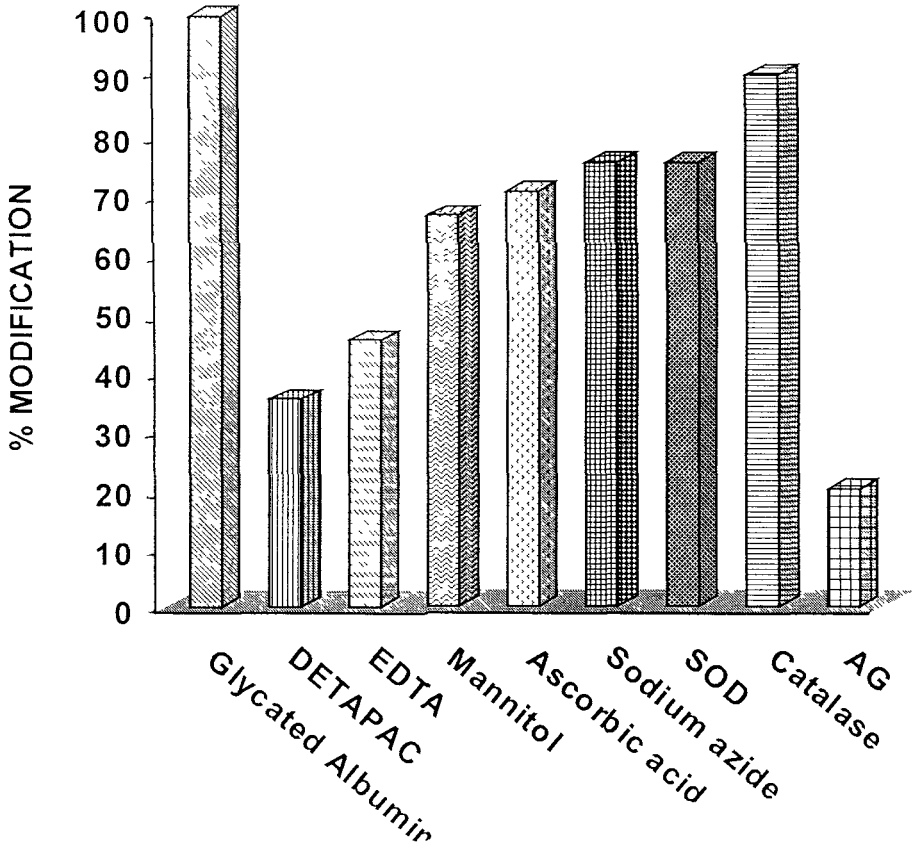


Fig. 13. Effect of free radical scavengers and antioxidants on the modification of HSA induced by glucose. The $^{\cdot}OH$ scavenger (mannitol 100 mM), $O_2^{\cdot -}$ scavanger (sodium azide 100 mM), antioxidant (ascorbic acid 5 mM) and metal ions chelators (DETAPAC 100 mM, EDTA 10mM). SOD and catalase (500 units/ml) and aminoguanidine (AG) were used at a concentration of 5 mM.

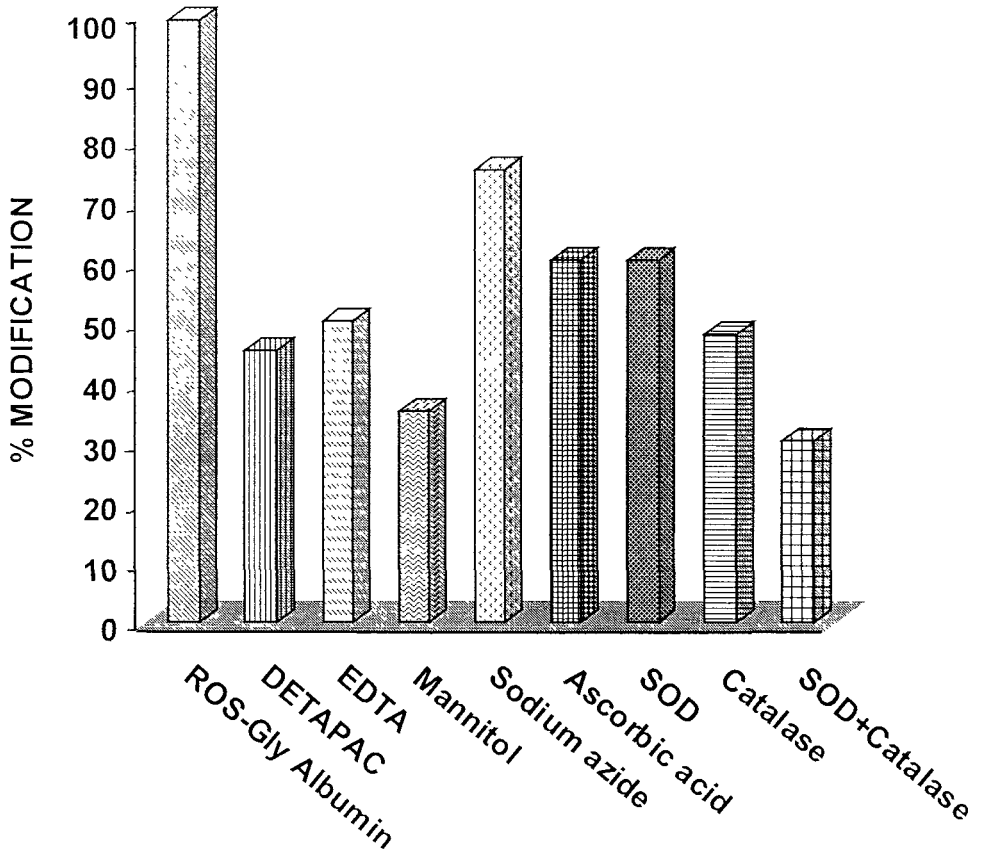


Fig. 14. Effect of free radical scavenger and antioxidant on the modification of glycated HSA induced by ROS. The $^{\cdot}OH$ scavengers (mannitol 100 mM), $O_2^{\cdot -}$ scavanger (100 mM), antioxidant (ascorbic acid 5 mM) and metal chelators (DETAPAC 100 mM, EDTA 10mM). SOD and catalase were used at 500 Units/ml.

Antibodies against native HSA

The antiserum showed a titer of at least 1:12800 when tested by direct binding ELISA (Fig. 15). Preimmune serum showed negligible binding. Induced antibodies were found to be specific for the immunogen. Inhibition ELISA showed a maximum of 70.4% inhibition (Fig. 16). Fifty percent inhibition was achieved with 11.8 μg/ml of native HSA. The raised antibodies were found to be precipitating as observed by immunodiffusion (Fig. 17), showed observable precipitating lines between native HSA and native HSA antiserum.

Purification and binding characterization of native HSA immune IgG

Immunoglobulin G was isolated from pre-immune and immune rabbit antiserum of native HSA by affinity chromatography on Protein A-Agarose column (Fig. 18). The purity of IgG was evaluated by SDS-polyacrylamide gel electrophoresis in absence of a reducing agent. The purified IgG migrated as a single band on 7.5% polyacrylamide gel upon electrophoresis (Fig. 18 inset).

Direct binding ELISA of the purified native HSA IgG showed strong reactivity towards immunogen (Fig. 19). Pre-immune IgG from rabbit as negative control showed negligible binding.

Band shift assay

The binding of native HSA to their immune IgG was further ascertained by band shift assay. An increasing in the amount of anti-native HSA IgG incubated with constant amount of antigen, for 2 hr at 37°C and overnight at 4°C, caused a propotional increase in the formation of high molecular weight immune complexes resulting in increase intensity of the immune complex. However, the amount of unbound HSA showed a proportional decrease in its intensity (Fig. 20).

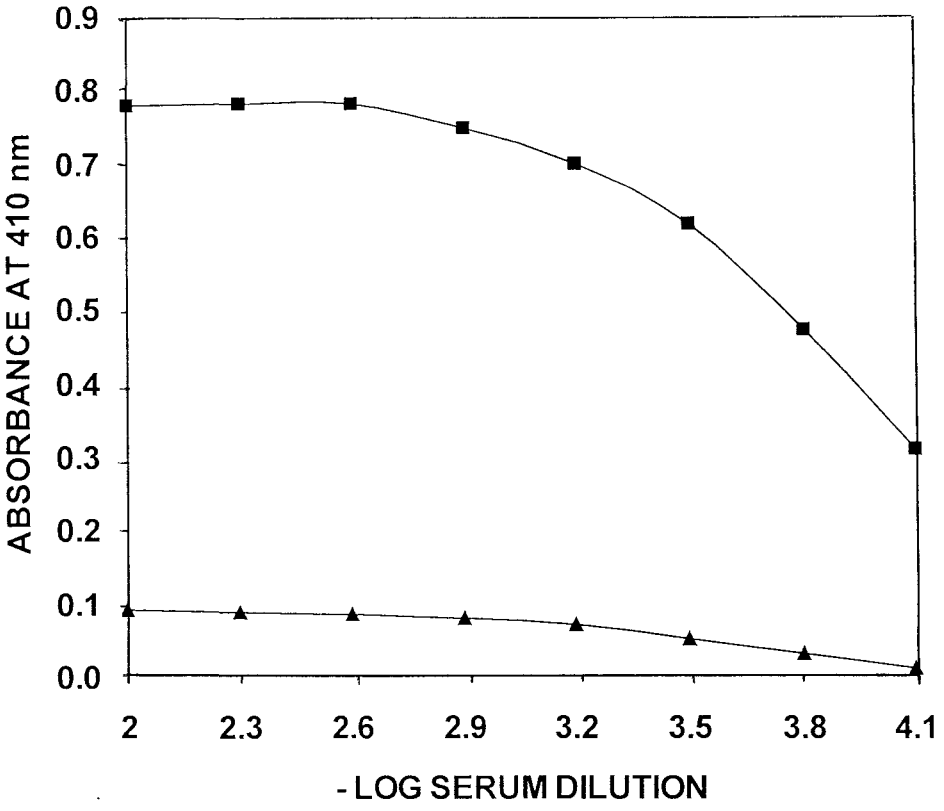


Fig. 15. Direct binding ELISA of native HSA with preimmune (▲) and immune (■) sera. Microtitre plates were coated with native HSA 20 μg/ml).

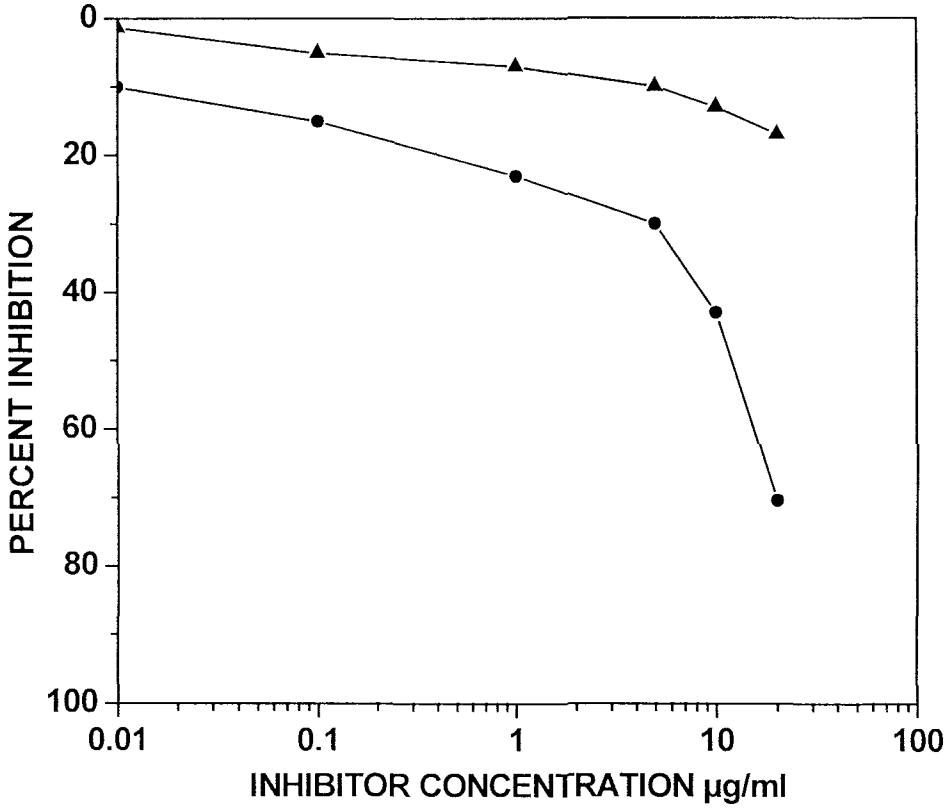


Fig. 16. Inhibition ELISA of anti-native HSA immune (■) and pre-immune (▲) sera with native HSA. Microtitre plates were coated with native HSA (20 µg/ml)

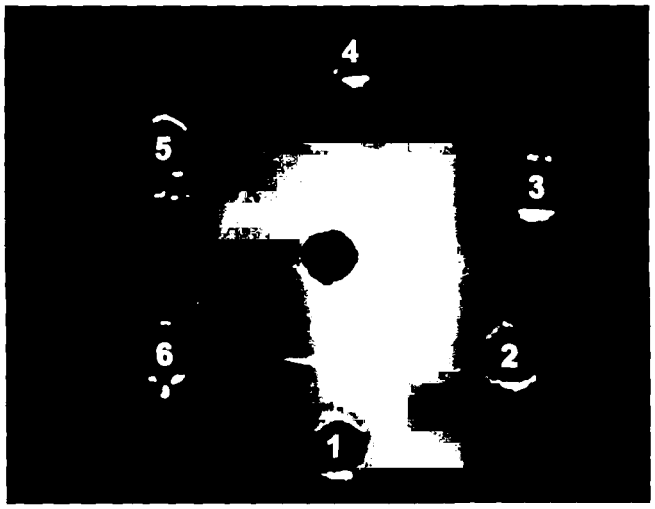


Fig. 17. Outcherlony double immunodiffusion of anti-native HSA antibodies with native HSA. Central well contains antigen whereas, well number 1 to 6 contain neat, 1:2, 1:4, 1:8, 1:16 and 1:32 same diluted serum, respectively.

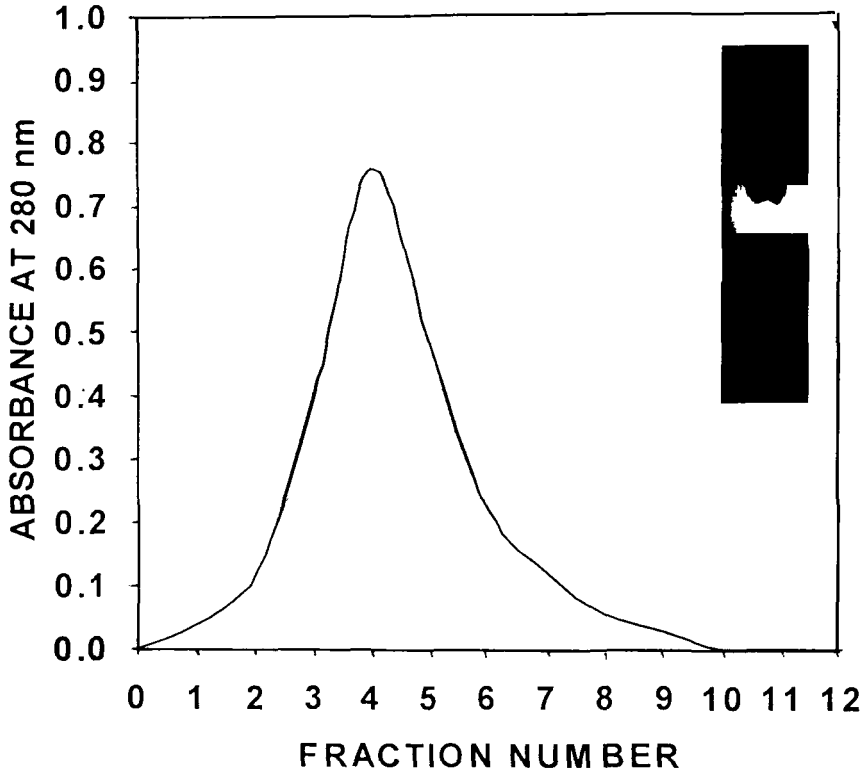


Fig. 18. Elution profile of anti–native HSA IgG on Protein-A Agarose column. **Inset:** SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.

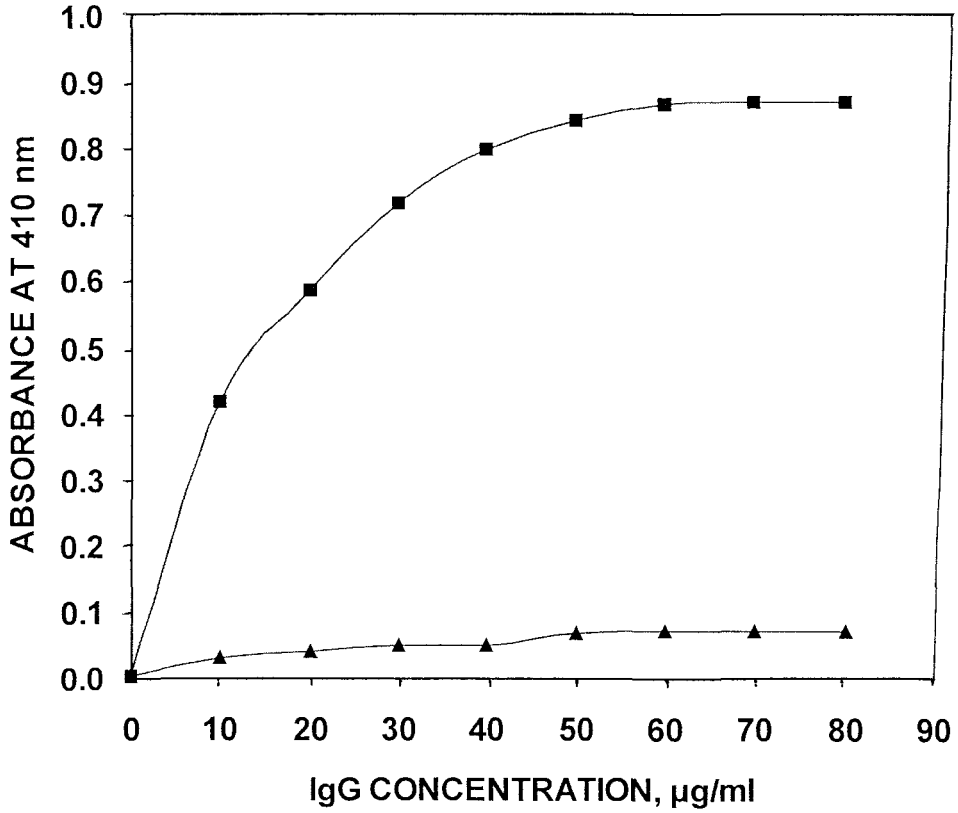


Fig. 19. Binding of affinity purified anti-native HSA immune IgG (■) and preimmune IgG (▲) to native HSA. Microtitre plates were coated with native HSA (20 µg/ml).

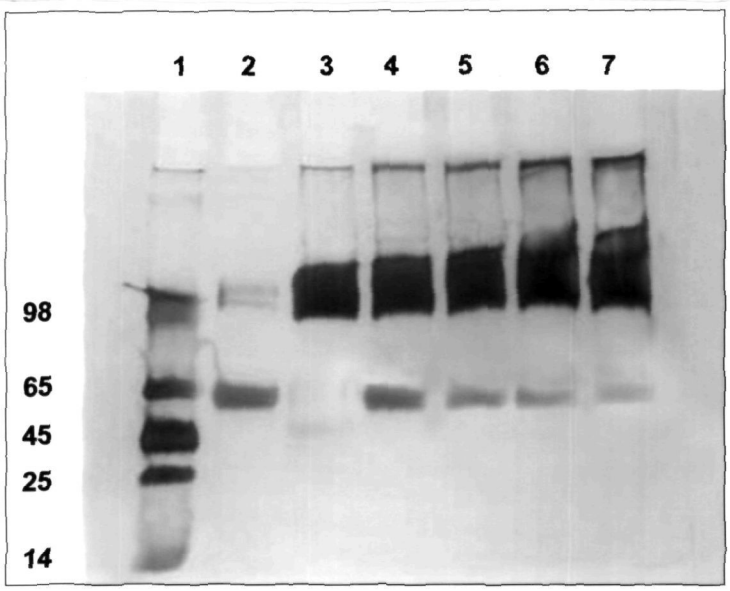


Fig. 20. Band shift assay of anti-native HSA IgG binding to native HSA. Native HSA (10 μg) (lane 2) and anti-native HSA IgG (10 μg) (lane 3) were incubated with buffer alone. Increasing amount of immune IgG (10, 20, 30 and 40 μg) with constant amount (10 μg) of native HSA through lanes 4 to 7, respectively were incubated for 2 hr at 37°C and overnight at 4 °C. Lane 1 contain protein molecular weight marker (98–14 kDa). Electrophoresis was carried out on 5.5% polyacrylamide gel for 3 hr at 50 V.

Antibodies against glycated HSA

Glycated HSA antiserum showed of high titre antibodies (> 1:12800) by direct binding ELISA. However, the binding of pre-immune serum was of low magnitude (Fig. 21). In competition ELISA, a maximum of 76% inhibition was observed at 20 μg/ml and 50% inhibition was achieved only at 7.8 μg/ml of immunogen (Fig. 22). The antibodies raised against glycated HSA was found to be precipitating and also showed fine precipitating line between glycated HSA and its antiserum, as observed by immunodiffusion (Fig. 23).

Purification and binding characterization of glycated HSA immune IgG

Immunoglobulin G was isolated from pre-immune and immune rabbit antiserum of glycated HSA by affinity chromatography on Protein A-Agarose column (Fig. 24). The purity of IgG was evaluated by SDS-polyacrylamide gel electrophoresis in absence of a reducing agent. The purified IgG migrated as a single band on 7.5% polyacrylamide gel upon electrophoresis (Fig. 24 inset).

Direct binding ELISA of the purified glycated HSA IgG showed strong reactivity towards its immunogen (Fig. 25). Pre-immune IgG from rabbit as negative control showed negligible binding.

Band shift assay

The binding of glycated HSA to their immune IgG was further ascertained by band shift assay. An increase in the amount of anti-glycated HSA IgG incubated with constant amount of antigen, for 2 hr at 37°C and overnight at 4°C, caused a proportional increase in the formation of high molecular weight immune complexes resulting in increase intensity of the immune complex. However, the amount of unbound HSA showed a proportional decrease in its intensity (Fig. 26).

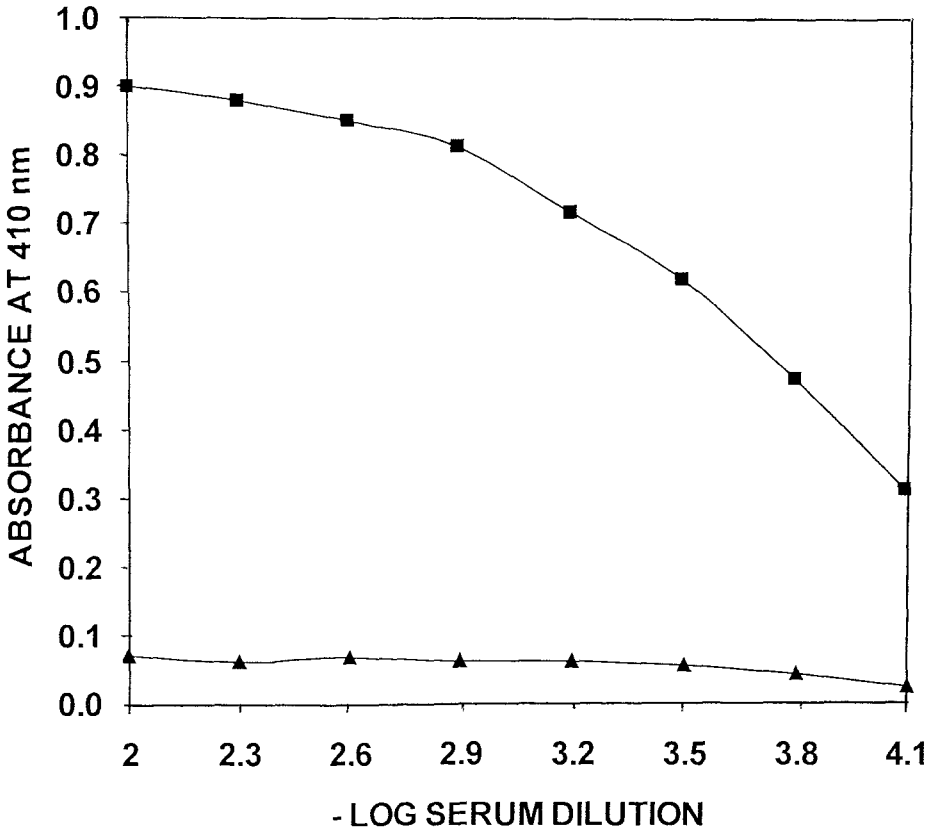


Fig. 21. Direct binding ELISA of glycated HSA with preimmune (▲) and immune sera (■). Microtitre plates were coated with glycated HSA (20 μg/ml).

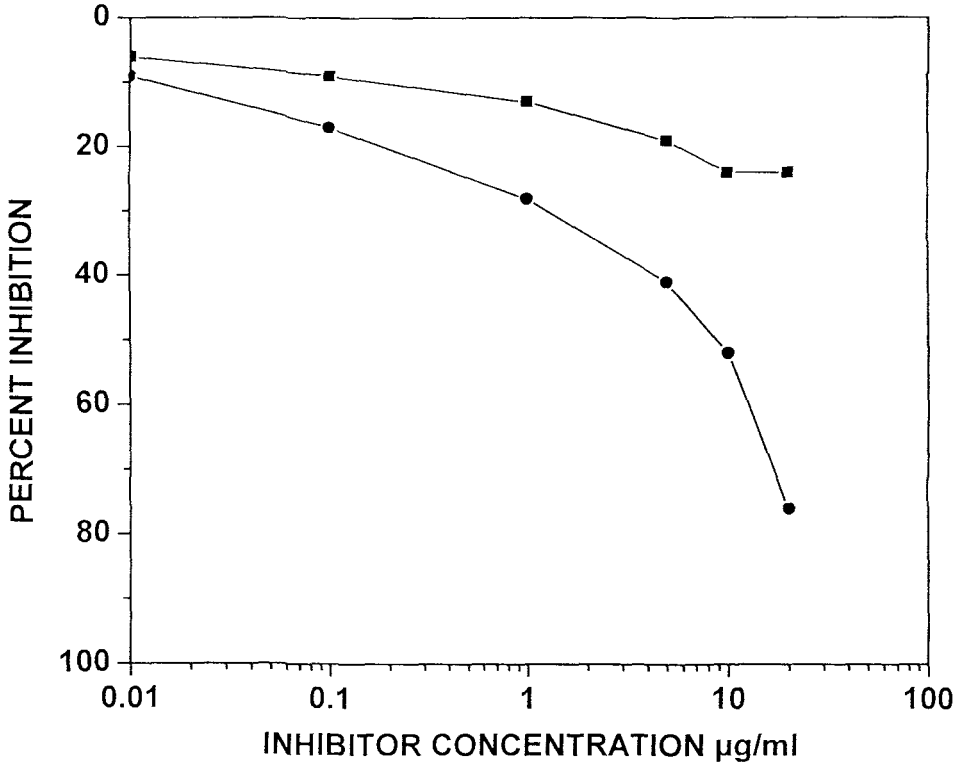


Fig. 22. Inhibition ELISA of anti-glycated HSA immune (●) and pre-immune (■) sera with glycated HSA. Microtitre plates were coated with glycated HSA (20 µg/ml)

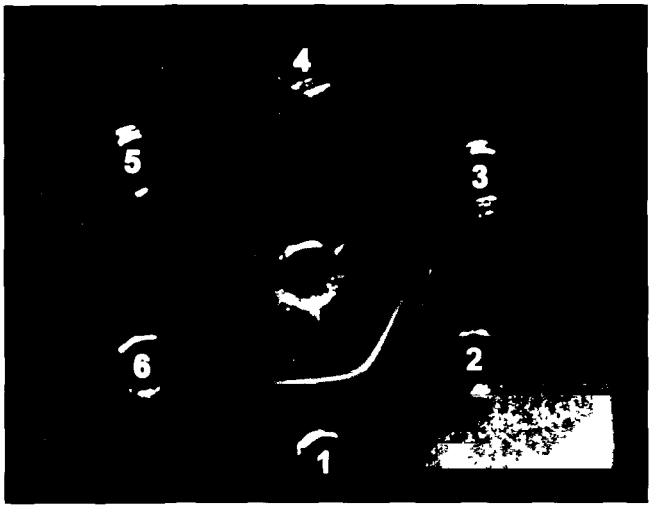


Fig. 23. Outcherlony double immunodiffusion of anti-glycated HSA antibodies with glycated HSA. Central well contains antigen whereas, well number 1 to 6 contain neat, 1:2, 1:4, 1:8, 1:16 and 1:32 same diluted serum, respectively.

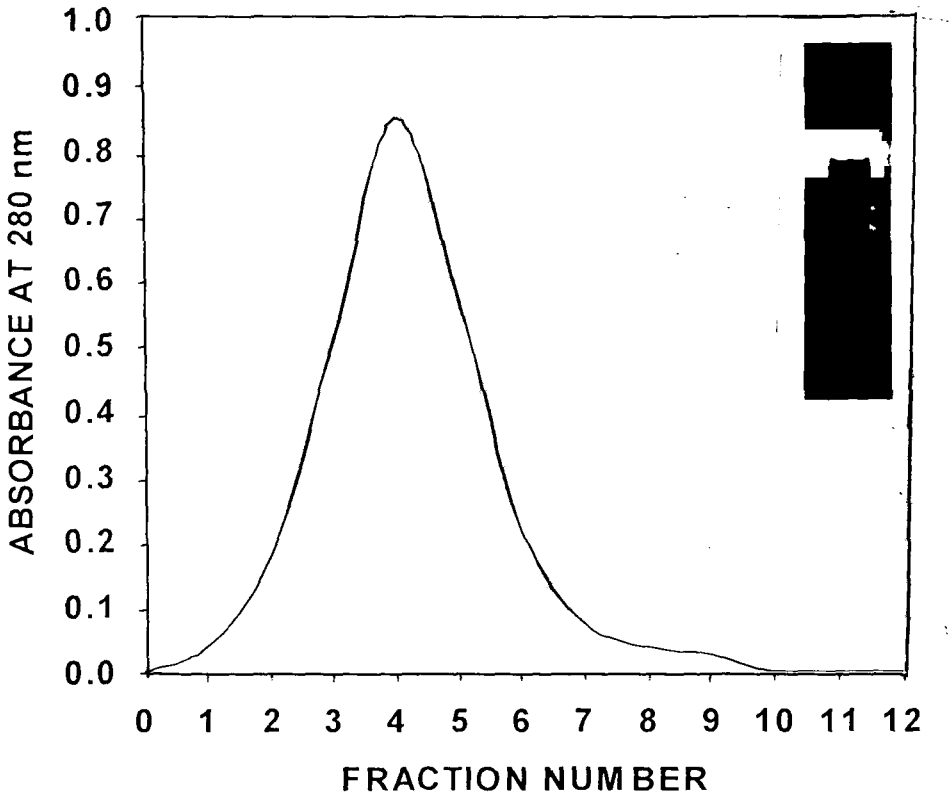


Fig. 24. Elution profile of anti–glycated HSA IgG on Protein-A Agarose column. **Inset:** SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.

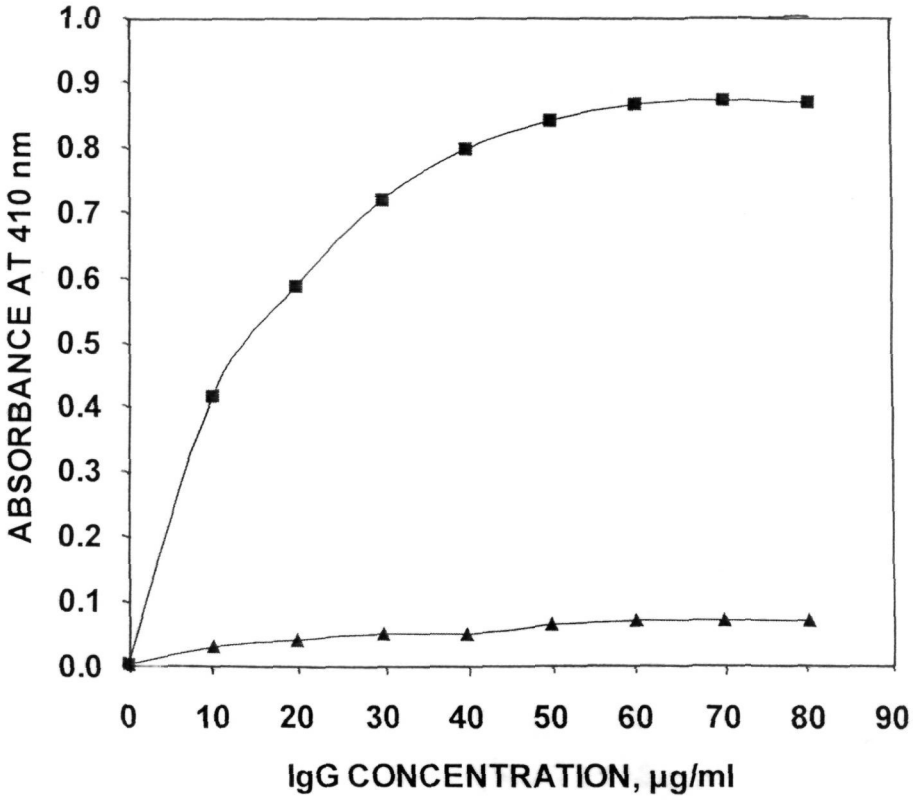


Fig. 25. Binding of affinity purified anti-glycated HSA immune IgG (■) and preimmune IgG (▲) to glycated HSA. Microtitre plates were coated with glycated HSA (20 µg/ml).

Fig. 26. Band shift assay of anti-glycated HSA IgG binding to glycated HSA. Glycated HSA (10 μg) (lane 2) and anti-native HSA IgG (10 μg) (lane 3) were incubated with buffer alone. Increasing amount of immune IgG (10, 20, 30 and 40 μg) with constant amount (10 μg) of native HSA through lanes 4 to 7, respectively were incubated for 2 hr at 37°C and overnight at 4°C. Lane 1 contain protein molecular weight marker (98–14 kDa). Electrophoresis was carried out on 5.5% polyacrylamide gel for 3 hr at 50 V.

Antibodies against ROS-glycated HSA

Direct binding ELISA was used to characterize the immune response in rabbit following immunization with ROS-glycated HSA. ROS-glycated HSA was highly immunogenic in rabbits. The antiserum showed a titre of at least 1:12800 when tested by direct binding ELISA (Fig. 27). Pre-immune serum served as negative control, did not show any appreciable binding to ROS-glycated HSA. The specificity of anti- ROS-glycated HSA antiserum was evaluated by competition ELISA. A maximum of 77.2% inhibition in antibody activity was obtained at an immunogen concentration of 20 μg/ml. The concentration of immunogen required for 50% inhibition was 5.8 μg/ml (Fig. 28). The raised antibody was found to be precipitating as observed by immunodiffusion (Fig. 29).

Purification and binding characterization of ROS-glycated HSA immune IgG

Immunoglobulin G was isolated from pre-immune and immune rabbit antiserum of ROS-glycated HSA by affinity chromatography on Protein A-Agarose column (Fig. 30). The purity of IgG was evaluated by SDS-polyacrylamide gel electrophoresis in absence of a reducing agent. The purified IgG migrated as a single band on 7.5% polyacrylamide gel upon electrophoresis (Fig. 30 inset).

Direct binding ELISA of the purified glycated HSA IgG showed strong reactivity towards its immunogen (Fig. 31). Pre-immune IgG from rabbit as negative control showed negligible binding.

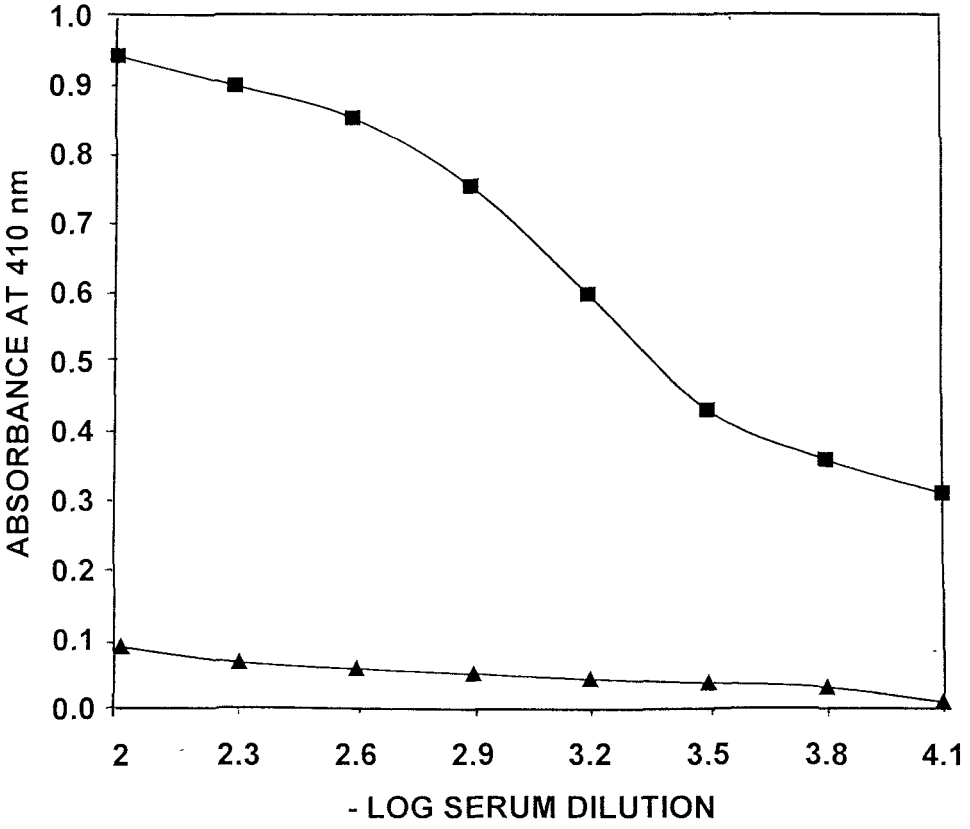


Fig. 27. Direct binding ELISA of ROS-glycated HSA with preimmune (▲) and immune sera (■). Microtitre plates were coated with ROS-glycated HSA (20 μg/ml).

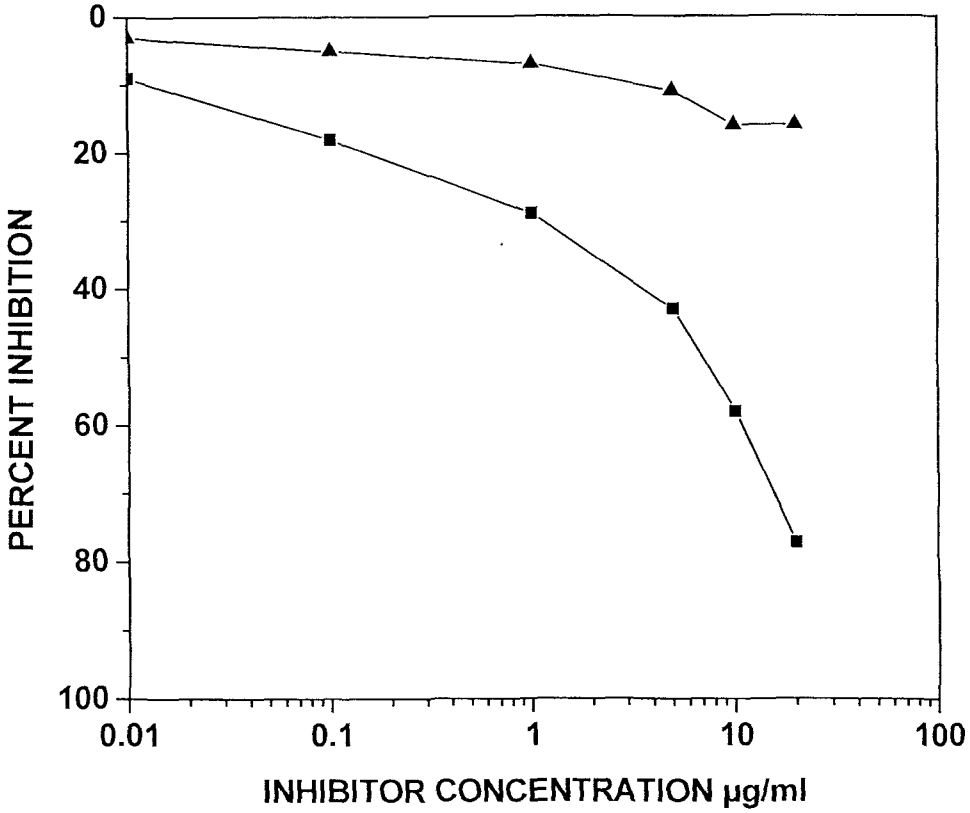


Fig. 28. Inhibition ELISA of anti-ROS-glycated HSA immune (■) and pre-immune (▲) sera with ROS-glycated HSA. Microtitre plates were coated with ROS-glycated HSA (20 µg/ml).

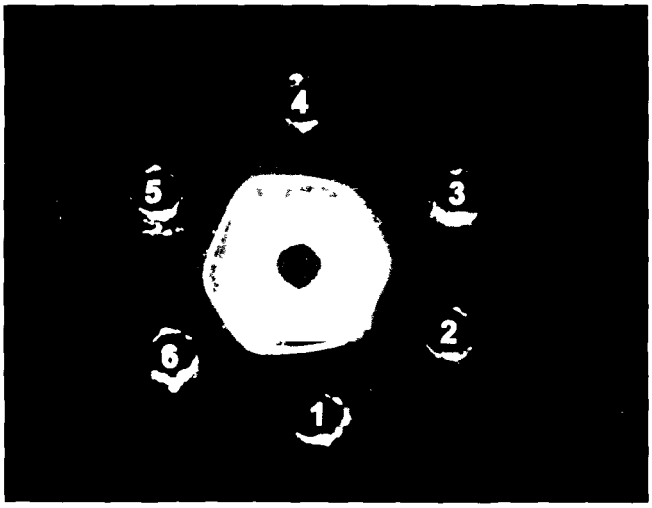


Fig. 29. Outcherlony double immunodiffusion of anti-ROS-glycated HSA antibodies with ROS-glycated HSA. Central well contains antigen whereas, well number 1 to 6 contain neat, 1:2, 1:4, 1:8, 1:16 and 1:32 diluted serum, respectively.

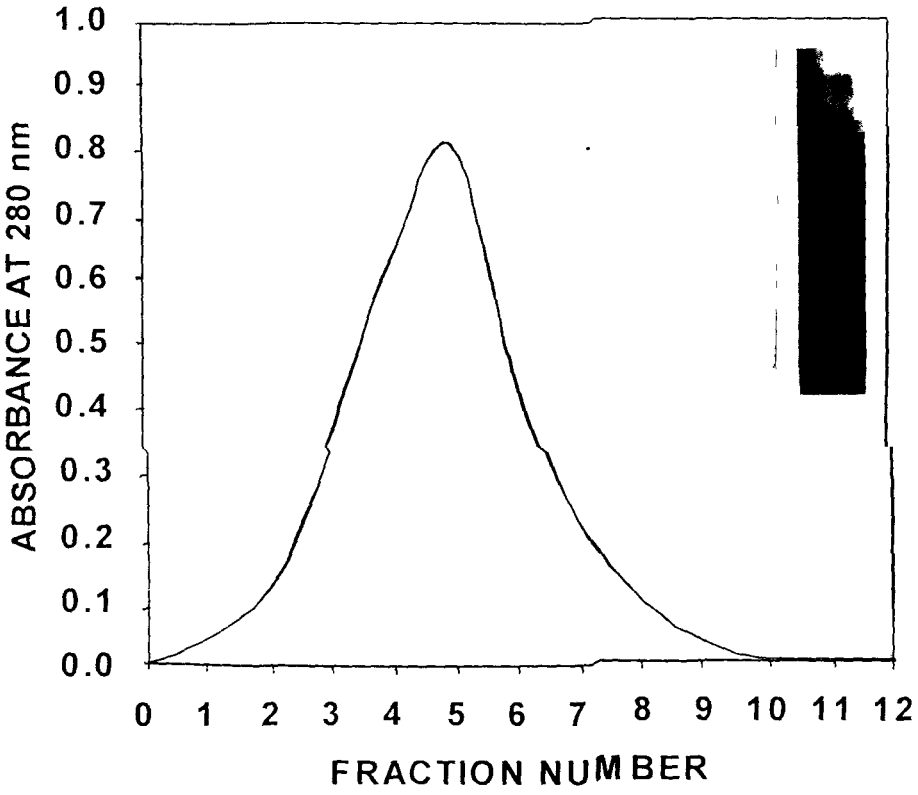


Fig. 30. Elution profile of anti–ROS-glycated HSA IgG on Protein-A Agaros column. **Inset:** SDS-PAGE of purified IgG on 7.5% polyacrylamic gel.

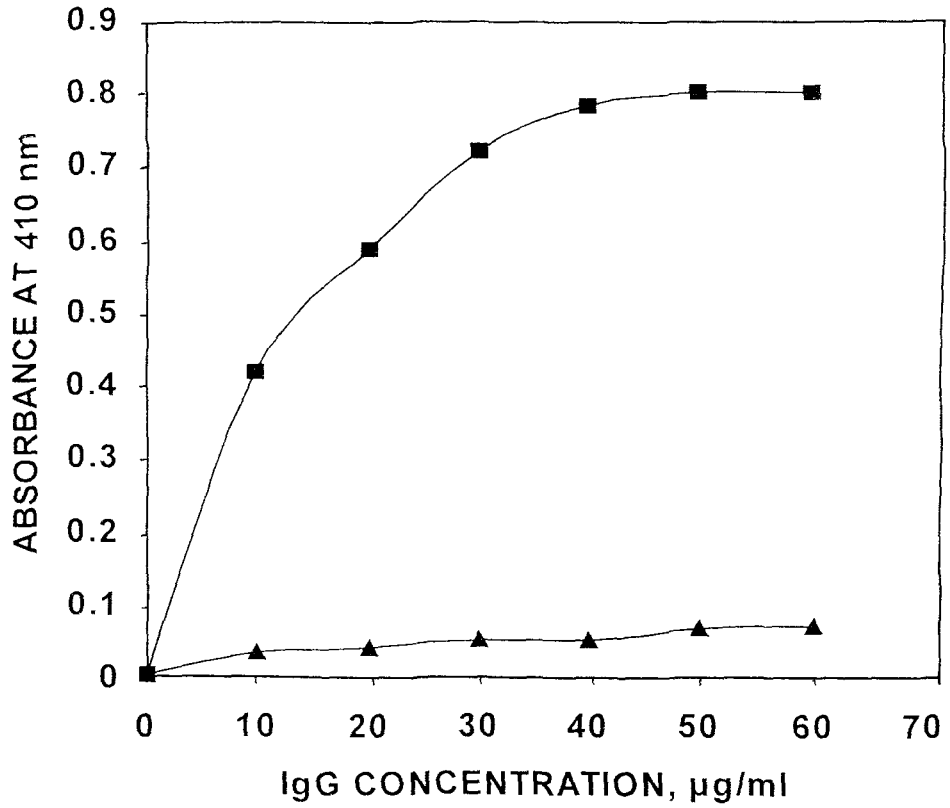


Fig. 31. Binding of affinity purified anti-ROS-glycated HSA immune IgG (■) and preimmune IgG (▲) to ROS-glycated HSA. Microtitre plates were coated with ROS-glycated HSA (20 µg/ml).

Band shift assay

The binding of ROS-glycated HSA to their immune IgG was further ascertained by band shift assay. An increasing amount of anti-ROS-glycated HSA IgG was incubated with constant amount of antigen, for 2 hr at 37°C and overnight at 4°C. A proportional increase in the formation of high molecular weight immune complexes was observed. No increase in the intensity of ICs with retarted mobility was evident. Moreover, the amount of unbound HSA showed a proportional decrease in its intensity (Fig. 32).

Immuno-cross reactivity of anti-native HSA antibodies

The antigenic specificity of the induced anti-native HSA antibodies was characterized by competitive inhibition assay using various inhibitors like blood proteins and nucleic acid (Figs. 33 - 35). A maximum of 78.0% inhibition of anti-native HSA antibody with immunogen as inhibitor was observed (Fig. 33a). Fifty percent inhibition was achieved with only 9.6 μg/ml of native HSA. The induced antibodies showed a broad spectrum of reactivity as observed by its binding to a variety of protein antigens. Glycated HSA and ROS-glycated HSA showed inhibitions of 40% and 23%, respectively (Fig. 33a). Native IgG, glycated IgG and ROS-glycated IgG showed inhibitions of 9.5%, 31.0% and 27%, respectively (Fig. 33b). Native BSA, glycated BSA and ROS-glycated BSA showed 37%, 35% and 33% inhibition, respectively (Fig. 34a). Native poly-L lysine, glycated poly-L lysine and ROS-glycated poly-L lysine showed 11.5%, 21% and 14.1% showed inhibition, respectively, at 20 μg/ml (Fig. 34b). Twenty weeks glycated HSA and ROS-HSA at 20 μg/ml showed 28.3% and 31.4% inhibition, respectively (Fig. 35a). Fructose modified HSA and native plasmid DNA at 20 μg/ml showed 27.1% and 19% inhibition, respectively (Fig. 35b). Table 3 summarizes the data of the binding characteristics of anti-native HSA IgG as determined by inhibition ELISA.

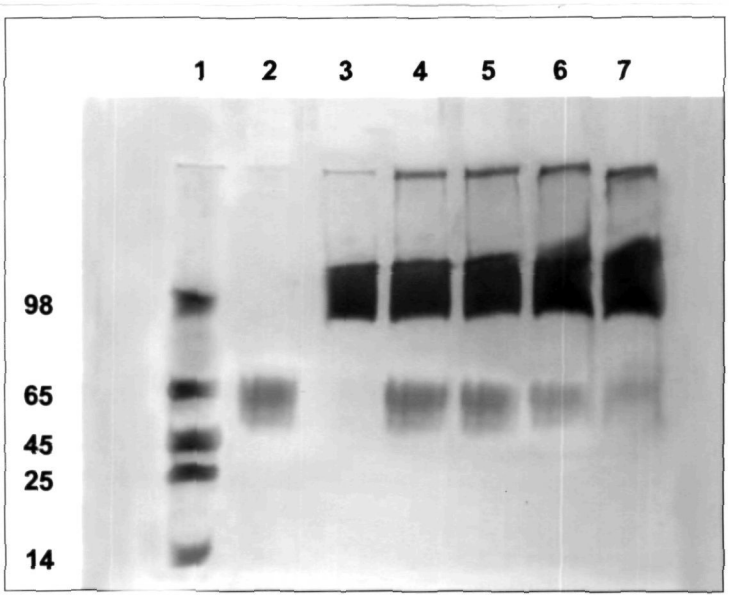


Fig. 32. Band shift assay of anti-ROS-glycated HSA IgG binding to ROS-glycated HSA. ROS-glycated HSA (10 μg) (lane 2) and anti-ROS-glycated HSA IgG (10 μg) (lane 3) were incubated with buffer alone. Increasing amount of immune IgG (10, 20, 30 and 40 μg) with constant amount (10 μg) of ROS-glycated HSA through lanes 4 to 7, respectively were incubated for 2 hr at 37°C and overnight at 4°C. Lane 1 contain protein molecular weight marker (9–14 kDa). Electrophoresis was carried out on 5.5% polyacrylamide gel for 3 hr at 50 V.

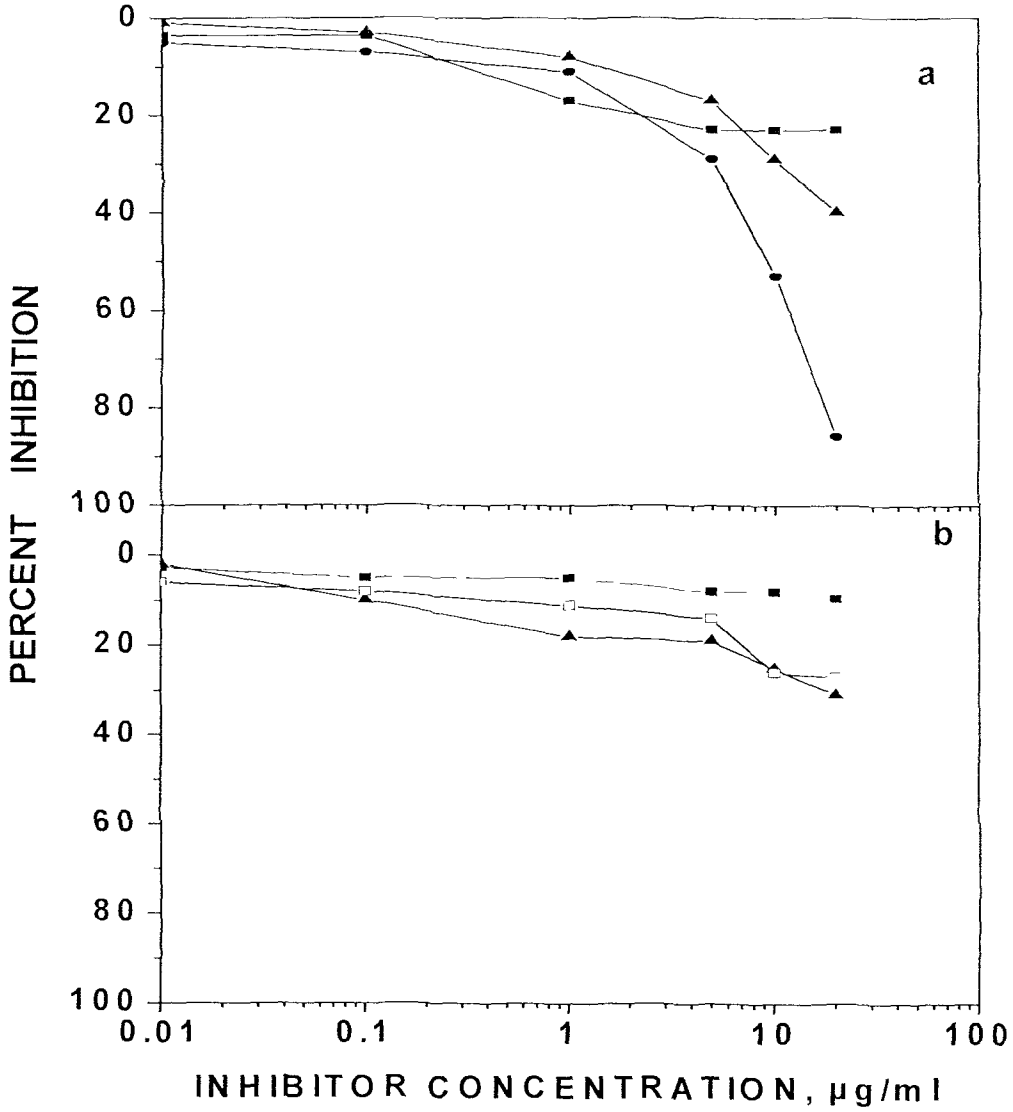


Fig. 33. Inhibition of anti-native HSA IgG binding to native HSA. Microtitre plates were coated with native HSA (20 µg/ml). The competitors were **(a)** native HSA (●), glycated HSA (▲) and ROS-glycated HSA (■). **(b)** native human IgG (■), glycated human IgG (▲) and ROS-glycated IgG (▫).

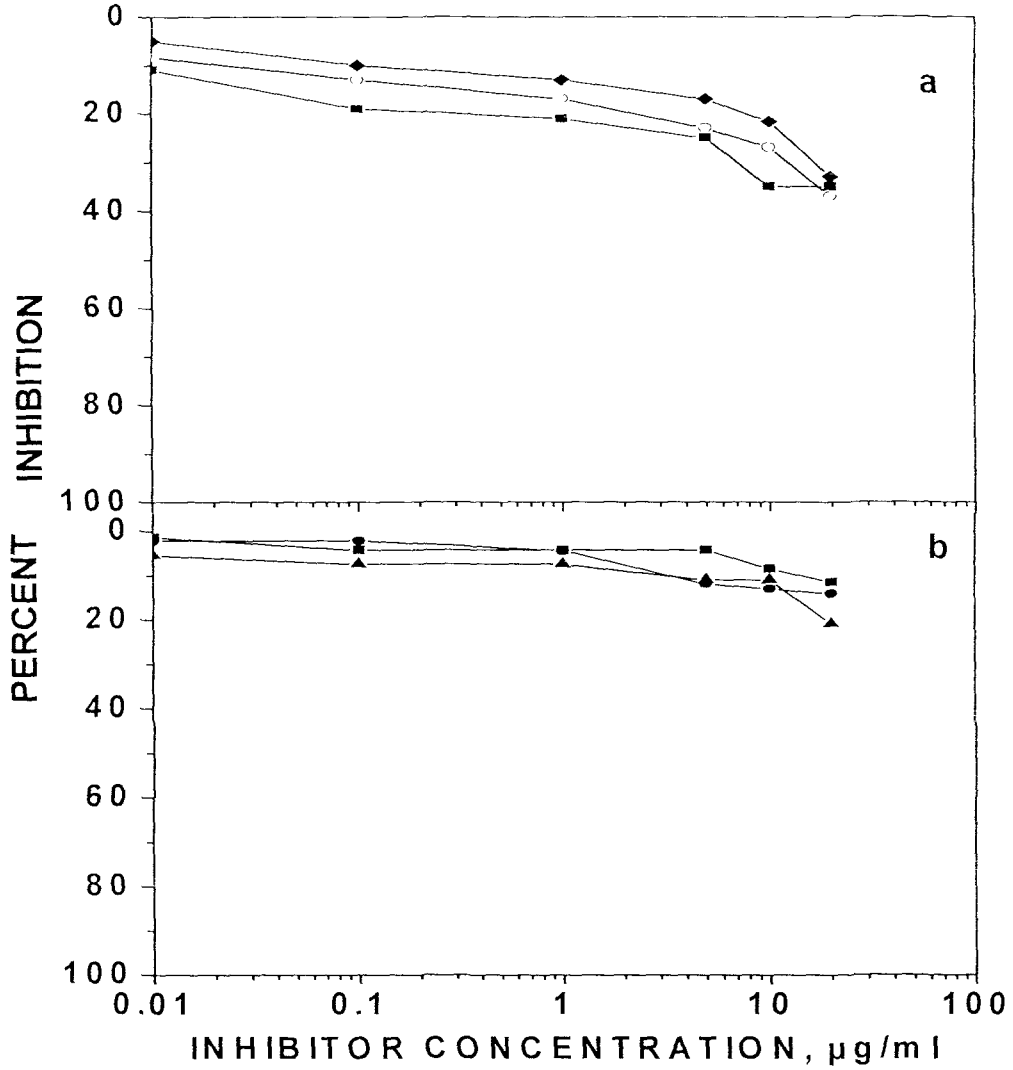


Fig. 34. Inhibition of anti-native HSA IgG binding to native HSA. Microtitre plates were coated with native HSA (20 µg/ml). The competitors were **(a)** native BSA (■), glycated BSA (▲) and ROS-glycated BSA (○), **(b)** native poly-L lysine (■), glycated poly-L lysine human (▲) and ROS-glycated poly-L lysine(●).

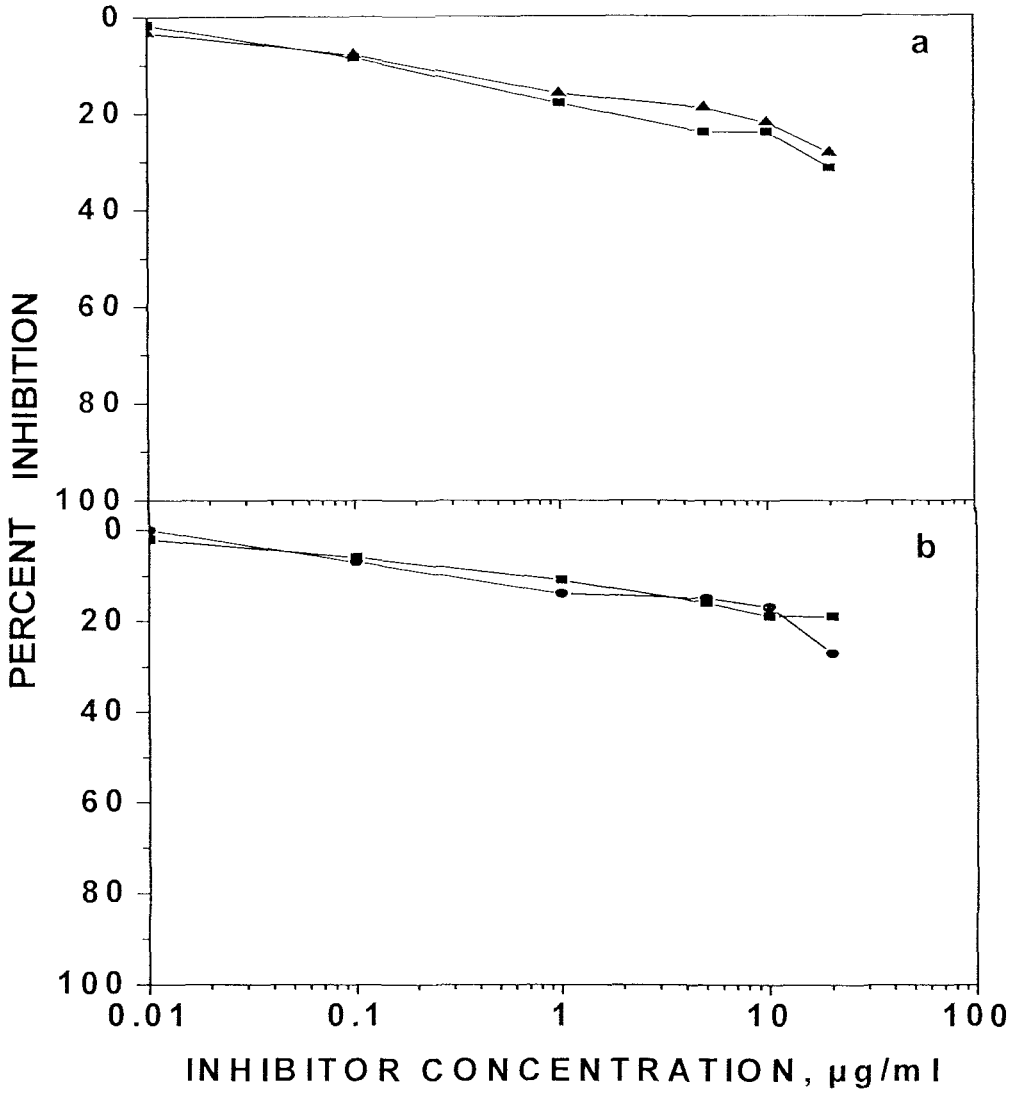


Fig. 35. Inhibition of anti-native IgG binding to native HSA. Microtitre plates were coated with native HSA (20 µg/ml). The competitors were **(a)** ROS-HSA (■), fructated HSA(▲), **(b)** glycated HSA (20 weeks) (●), native plasmid DNA(■).

TABLE 3

Antigenic specificity of anti-native HSA antibodies

Inhibitors	Maximum % inhibition at 20 µg/ml
Native HSA	78.0
Glycated HSA	40.0
ROS-glycated HSA	23 0
Native human IgG	9 5
Glycated human IgG	31.0
ROS-glycated human IgG	27.0
Native BSA	37.0
Glycated BSA	35.0
ROS-glycated BSA	33 0
Poly-L lysine	11.5
Glycated poly-L lysine	21.0
ROS-glycated poly-L lysine	14.1
ROS-HSA	31.4
Fructated HSA	27.1
Glycated HSA (20 weeks)	28.3
Plasmid DNA	19.0

ELISA plates were coated with native HSA at 20 µg/ml

Immuno-cross reactivity of anti-glycated HSA antibodies

The antigenic specificity of the induced anti-glycated HSA antibodies was characterized by competitive inhibition assay using the immunogen, blood proteins, and nucleic acid as inhibitors (Figs. 36-38). A maximum of 88.1% inhibition of antibody binding with immunogen as inhibitor was observed (Fig. 36a). Fifty percent inhibition was achieved with only 6 μg/ml of glycated HSA. The induced antibodies showed a broad spectrum of reactivity as observed by its binding to a variety of protein antigens. Native HSA and ROS-glycated HSA showed inhibitions of 30% and 38% (Fig. 36a). Native IgG, glycated IgG and ROS-glycated IgG showed inhibitions of 7.6%, 56% and 22.1%, respectively (Fig. 36b). Native BSA, glycated BSA and ROS-glycated BSA showed 21.8%, 50.2% and 39.1% inhibition, respectively (Fig. 37a). Native poly-L lysine, glycated poly-L lysine and ROS-glycated poly-L lysine showed 17.1%, 43% and 31% inhibition, respectively, at 20 μg/ml (Fig. 37b). Twenty weeks glycated HSA showed a significant inhibition and fifty percent inhibition was achieved with 10.8 μg/ml of twenty weeks glycated HSA (Fig. 38a). ROS-HSA at 20 μg/ml showed 29.2% inhibition (Fig. 38a). Fructose modified HSA and native plasmid DNA at 20 μg/ml showed 52.1% and 23% inhibition, respectively (Fig. 38b). The inhibition data of anti-glycated HSA antibodies with various inhibitors are summarized in Table 4.

Immuno-cross reactivity of anti-ROS-glycated HSA antibodies

Figures 39 to 41 showed inhibition studies of anti-ROS-glycated HSA antibodies with various inhibitors. A maximum of 90.1% inhibition in the binding of antibodies with immunogen as inhibitor was observed, concentration of immunogen required for fifty percent inhibition was only 8.3 μg/ml (Fig. 39a). Native HSA and glycated HSA showed 28.9% and 31.3% inhibition at 20 μg/ml, respectively, (Fig. 39a). Native IgG and glycated IgG showed less inhibition of 19.3% and 16.5%, respectively (Fig. 39b).

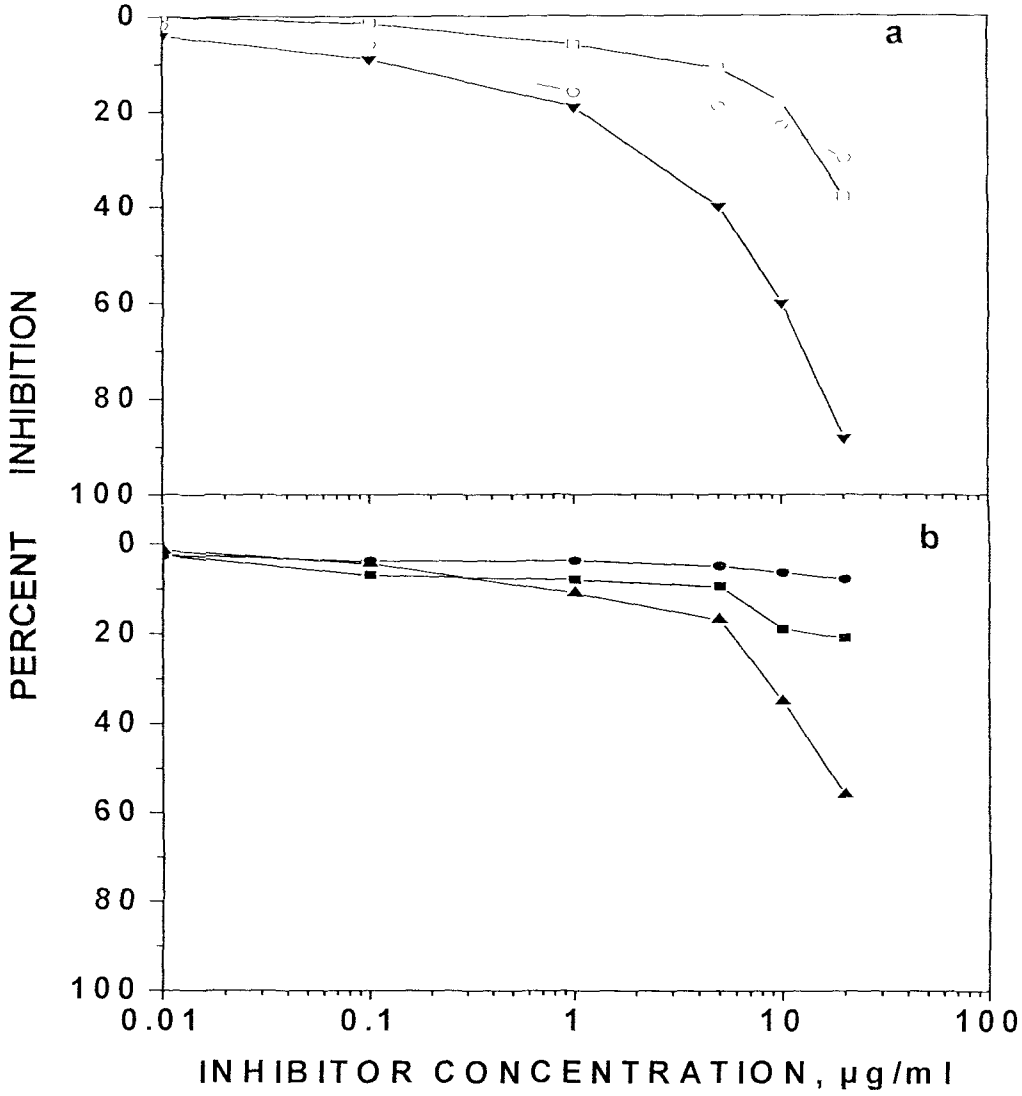


Fig. 36. Inhibition of anti-glycated IgG binding to glycated HSA. Microtitre plates were coated with glycated HSA (20.0 µg/ml). The competitors were **(a)** native HSA (○), glycated-HSA (▼) and ROS-glycated HSA (⊟), **(b)** native human IgG (●), glycated human IgG (▲) and ROS-gly IgG (■).

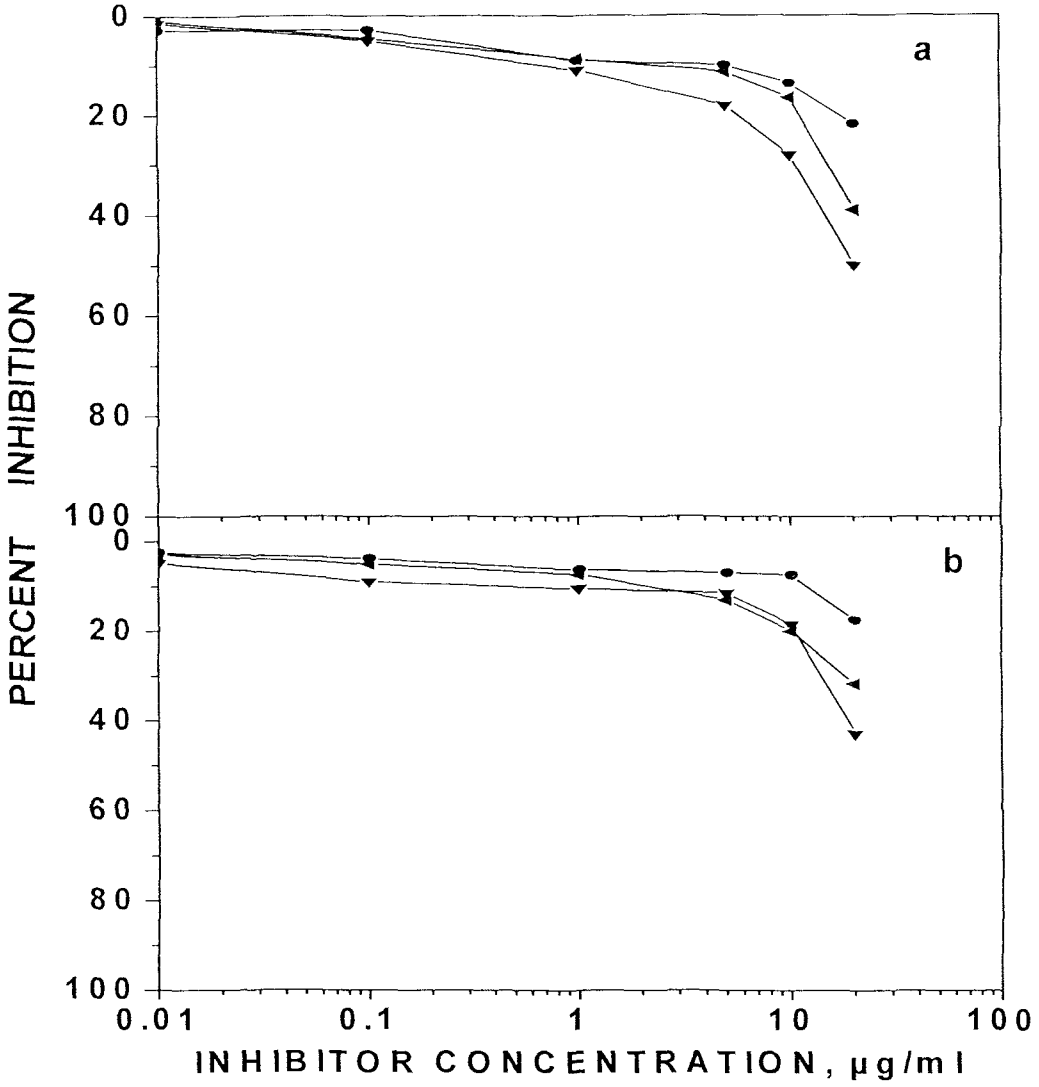


Fig. 37. Inhibition of anti-glycated HSA IgG binding to glycated HSA. Microtitre plates were coated with glycated HSA (20 µg/ml). The competitors were (a) native BSA (●), glycated BSA (▼) and ROS-glycated BSA (▲). (b) native poly-L lysine (●), glycated poly-L lysine (▲) and ROS-glycated poly-L lysine (▼).

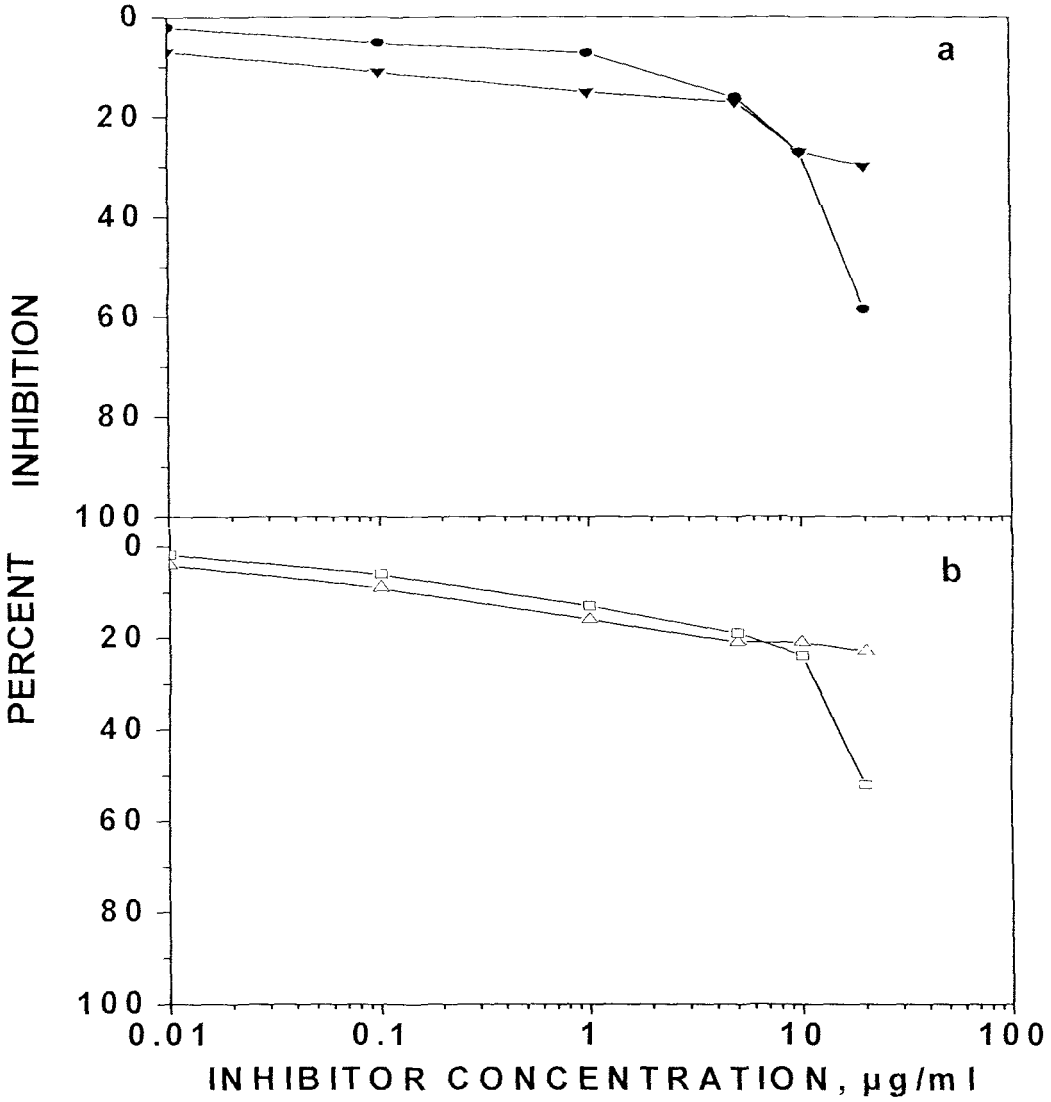


Fig. 38. Inhibition of anti-glycated HSA IgG binding to glycated HSA. Microtitre plates were coated with glycated HSA (20.0 µg/ml). The competitors were **(a)** glycated HSA (20 weeks) (●), fructated HSA (▼) and **(b)** ROS-HSA (□), native plasmid DNA (Δ).

TABLE 4

Antigenic specificity of anti-glycated HSA antibodies

Inhibitors	Maximum % inhibition at 20 µg/ml
Native HSA	30.0
Glycated HSA	88.1
ROS-glycated HSA	38.0
Native human IgG	7.6
Glycated human IgG	56.0
ROS-glycated human IgG	22.1
Native BSA	21.8
Glycated BSA	50.2
ROS-glycated BSA	39.1
Poly-L lysine	17.1
Glycated poly-L lysine	43.0
ROS-glycated poly-L lysine	31.0
Glycated HSA (20 weeks)	58.6
Fructated HSA	52.1
ROS-HSA	29.2
Native plasmid DNA	23.0

ELISA plates were coated with glycated HSA at 20 µg/ml

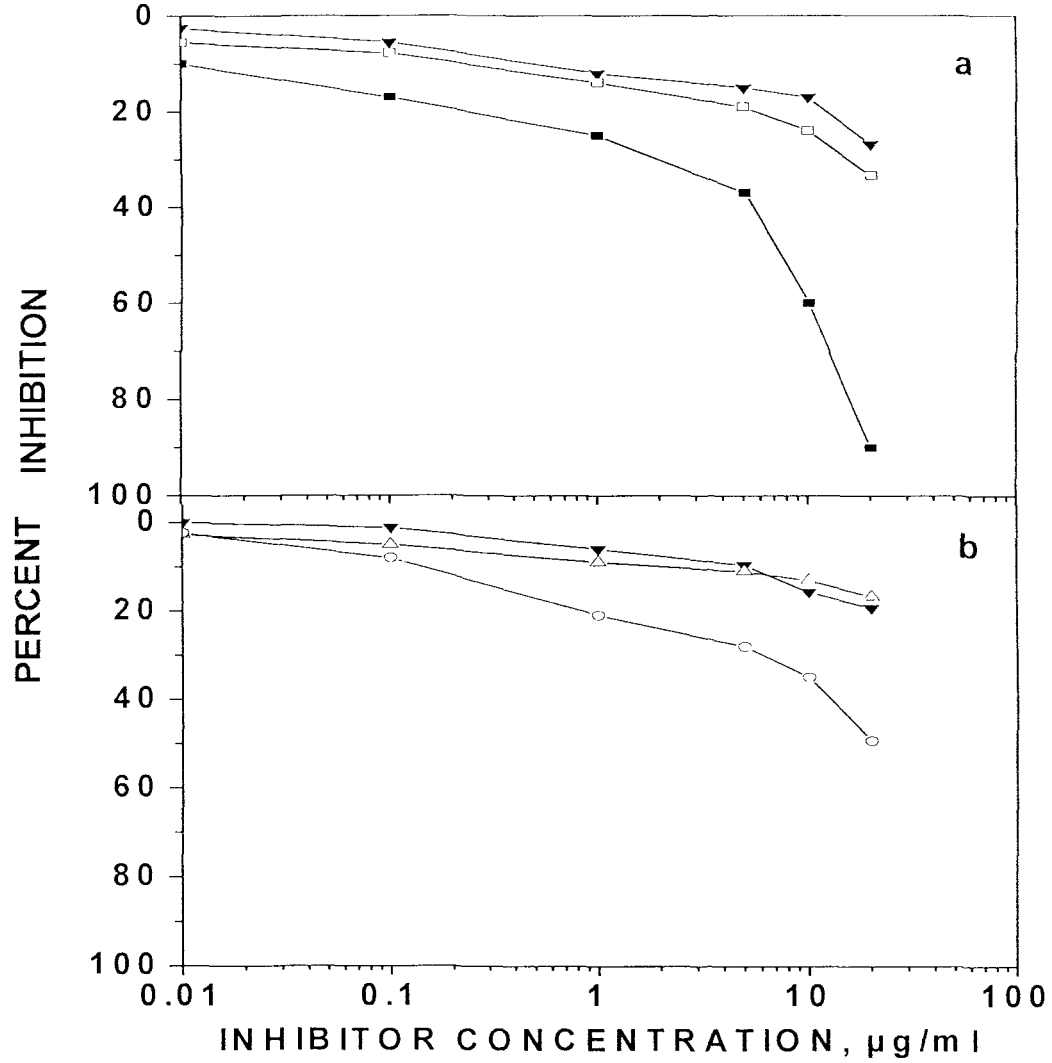


Fig. 39. Inhibition of anti-ROS-glycated IgG binding to ROS-glycated HSA. Microtitre plates were coated with ROS-glycated HSA (20.0 µg/ml). The competitors were **(a)** native HSA (▼), glycated-HSA (⧈) and ROS-glycated HSA (■), **(b)** native human IgG (Δ), glycated human IgG (■) and ROS-glycated IgG (○).

In contrast, ROS-glycated IgG was a potent inhibitor, showed a maximum inhibition of 49.3%. While native BSA and glycated BSA showed minimum inhibition of 14.6% and 16.7%, respectively. ROS-glycated BSA showed significant inhibition of (55%) (Fig. 40a). Fifty percent was achieved with 15 μg/ml of ROS-glycated HSA. Native poly L lysine, glycated poly L lysine and ROS-glycated poly L lysine showed 11.3%, 25% and 42.8 inhibition, respectively, at 20 μg/ml (Fig. 40b).

Twenty weeks glycated HSA and ROS-HSA at 20 μg/ml showed 14.6% and 35.3% inhibition, respectively (Fig. 41a). Fructose modified HSA and native plasmid DNA at 20 μg/ml showed 14.8% and 9% inhibition, respectively (Fig. 41b)

Antibodies in diabetic patients' sera against native and modified HSA

Sera from diabetic patients were tested for binding to native and modified HSA (glycated HSA and ROS-glycated HSA) by direct binding and competitive assay. The study comprised 24 sera from patients suffering from uncontrolled hyperglycemia (both types of diabetes mellitus) for long duration. The sera from nine diabetic patients having secondary complications like retinopathy, nephropathy and diabetic arteriosclerosis have also been studied. Sera from normal or healthy individuals served as controls. Diabetic sera were obtained after careful clinical examination of patients which proven positive glucose tolerance test attending J. N. Medical College Hospital, A. M. U. Aligarh.

Diabetic patient's sera showed appreciable binding to glycated HSA and ROS-glycated HSA as compared to native HSA. Similar results were obtained with the sera of diabetic patients having secondary complications (Fig. 42). These patients showed greater recognition to ROS-glycated HSA as compared to glycated HSA.

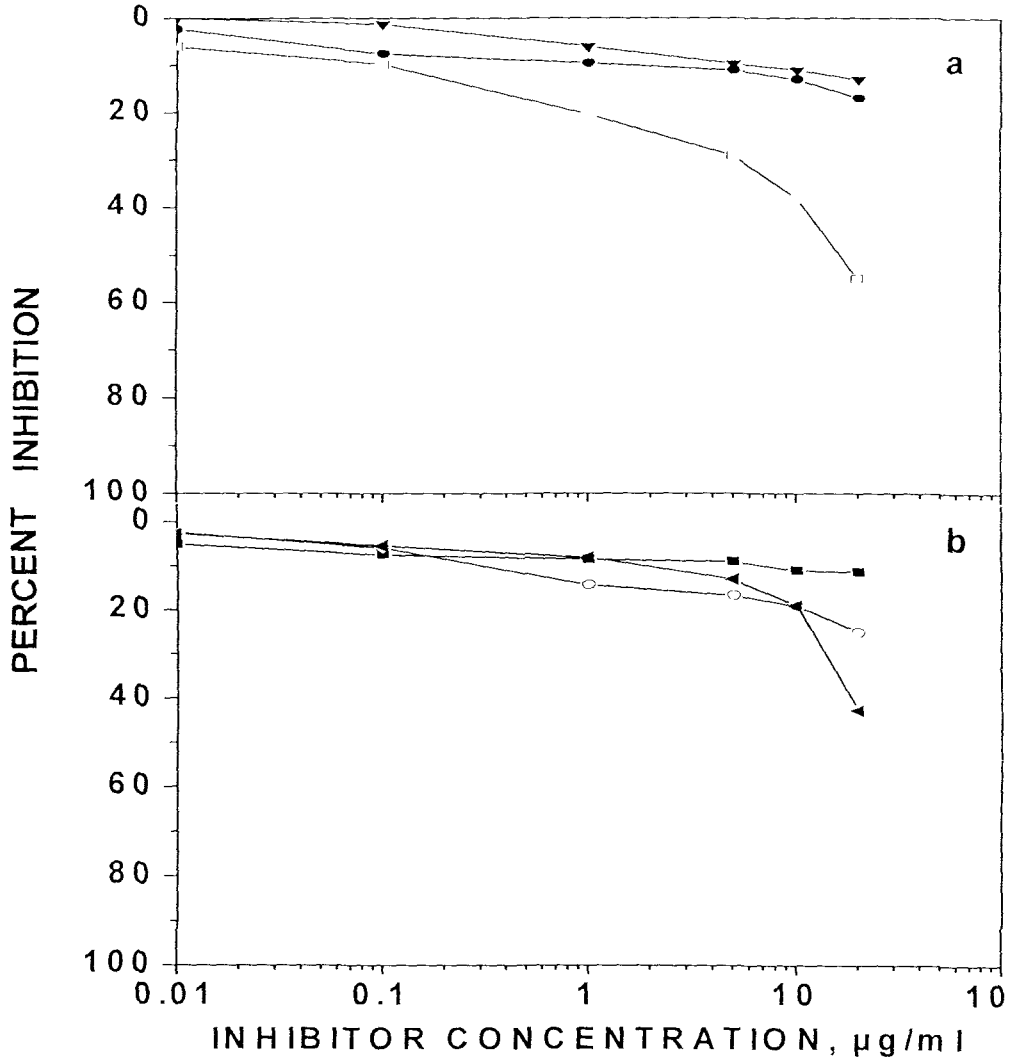


Fig. 40. Inhibition of anti-ROS-glycated HSA IgG binding to ROS-glycated HSA. Microtitre plates were coated with ROS-glycated HSA (20 µg/ml). The competitors were **(a)** native BSA (▼), glycated BSA (●) and ROS-glycated BSA (⊟), **(b)** native poly-L lysine (■), glycated poly-L lysine (○) and ROS-glycated poly-L lysine (▲).

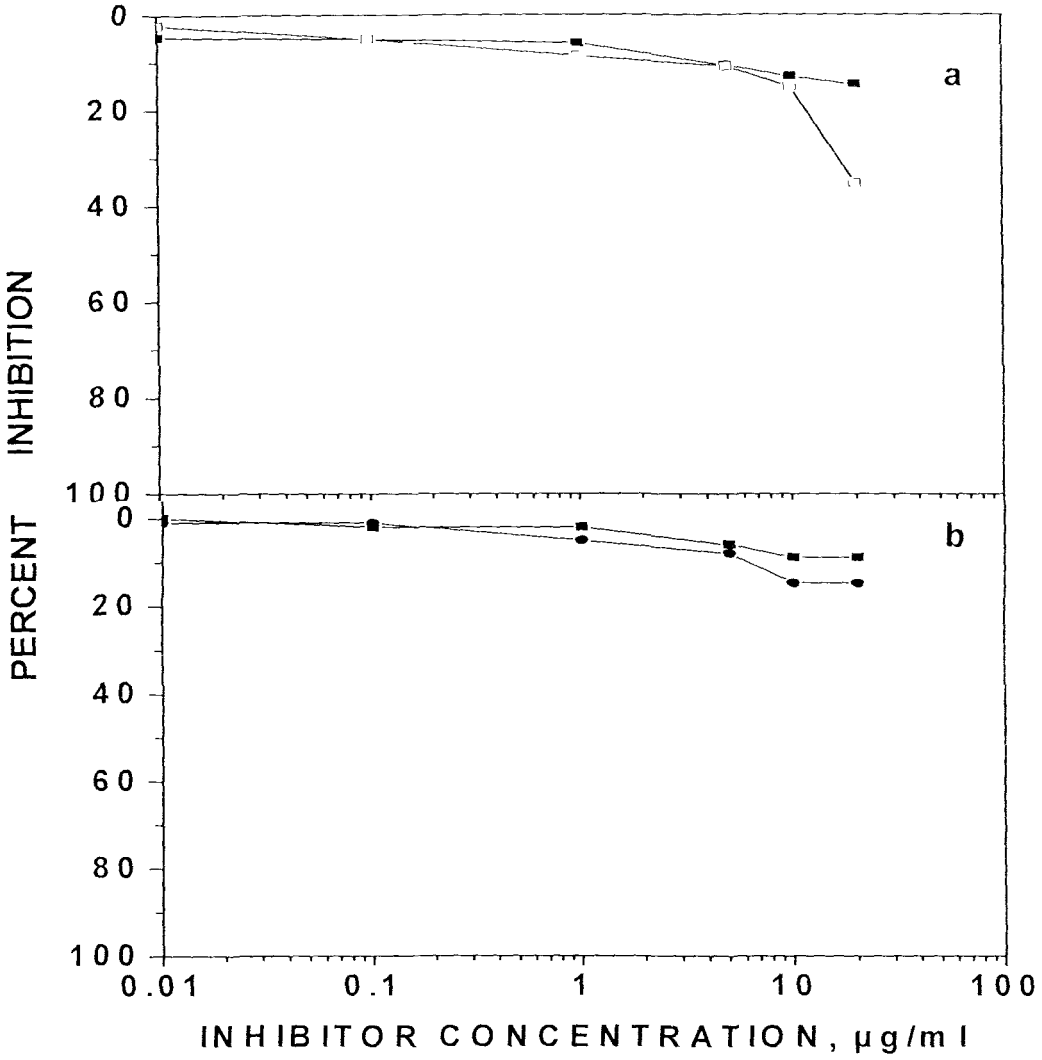


Fig. 41. Inhibition of anti-ROS-glycated HSA IgG binding to ROS-glycated HSA. Microtitre plates were coated with ROS-glycated HSA (20.0 µg/ml). The competitors were **(a)** glycated HSA (20 weeks) (●), ROS-HSA (□), **(b)** fructated HSA (●) and native plasmid DNA (■).

TABLE 5

Antigenic specificity of anti-ROS-glycated HSA antibodies

Inhibitors	Maximum % inhibition at 20 µg/ml
Native HSA	28.0
Glycated HSA	31.3
ROS-glycated HSA	90.1
Native human IgG	19.3
Glycated human IgG	16.5
ROS-glycated human IgG	49.3
Native BSA	13.0
Glycated BSA	16.7
ROS-glycated BSA	55.0
Poly-L lysine	11.3
Glycated poly-L lysine	25.0
ROS-glycated poly-L lysine	42.8
Glycated HSA (20 weeks)	14.6
ROS-HSA (20 weeks)	35.3
Fructated HSA	14.8
Native plasmid DNA	9.0

ELISA plates were coated with ROS-glycated HSA at 20 µg/ml

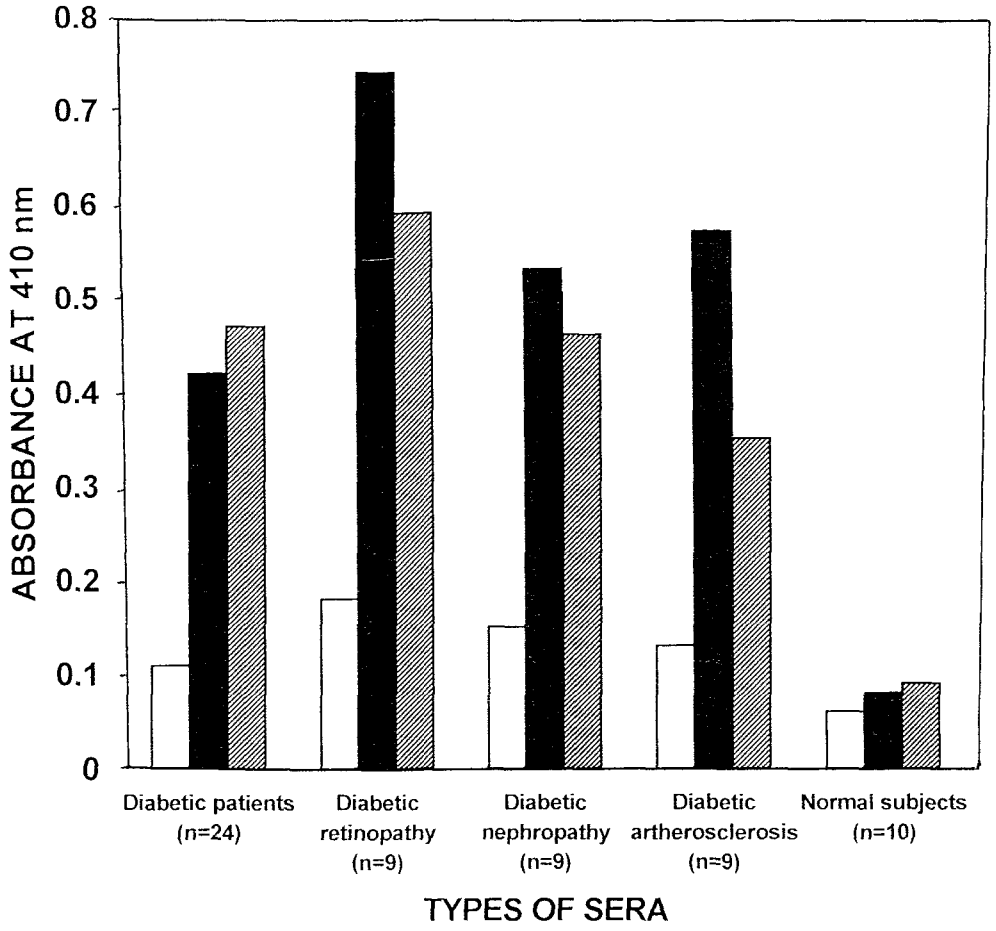


Fig. 42. Binding of various diabetic patients' sera to native HSA (□), glycated HSA (■) and ROS-glycated HSA (▨). Normal human sera served as control. The histogram shows the mean absorbance values of the normal and the various diabetic patients' sera.

However, glycated HSA showed greater binding than ROS-glycated HSA in sera of diabetic patients having secondary complications. Normal human sera showed less or negligible binding to native and modified HSA.

Competition ELISA was carried out to analyze the specific recognition of circulating autoantibodies in diabetic patients for native and modified HSA. The specific binding of the autoantibodies was remarkably higher for modified HSA ($p < 0.001$) than native HSA in all the sera tested.

Twenty four sera from diabetic patients', the observed maximum inhibition with native and glycated HSA was in the range of 16% to 37% and 32.9% to 59.3%, respectively, (Figs. 43- 48). The inhibition data of native and glycated HSA with diabetic patients' sera are summarized in Table 6.

In the case of diabetic patients having secondary complications, the observed inhibition at 20 μg/ml with native HSA and glycated HSA was in the range of be 16% to 33% and 54.1% to 68%, respectively (Figs. 49-51). The maximum inhibition (68%) was found in the patient of diabetic retinopathy (Fig. 49b). The data of patients' sera having secondary complications exhibited significant inhibition ($p < 0.001$) with glycated HSA as compared to native HSA and are summarized in Table 7.

The specificity of twenty four diabetic patients sera for native and ROS-glycated HSA was evaluated by inhibition ELISA (Fig. 52- 57). Native HSA showed maximum inhibitions ranging from 13% to 33.1%, ROS-glycated HSA showed high percent inhibition ranging from 31% to 62%. The inhibition data of native and ROS-glycated HSA with diabetic patients' sera are summarized in Table 8.

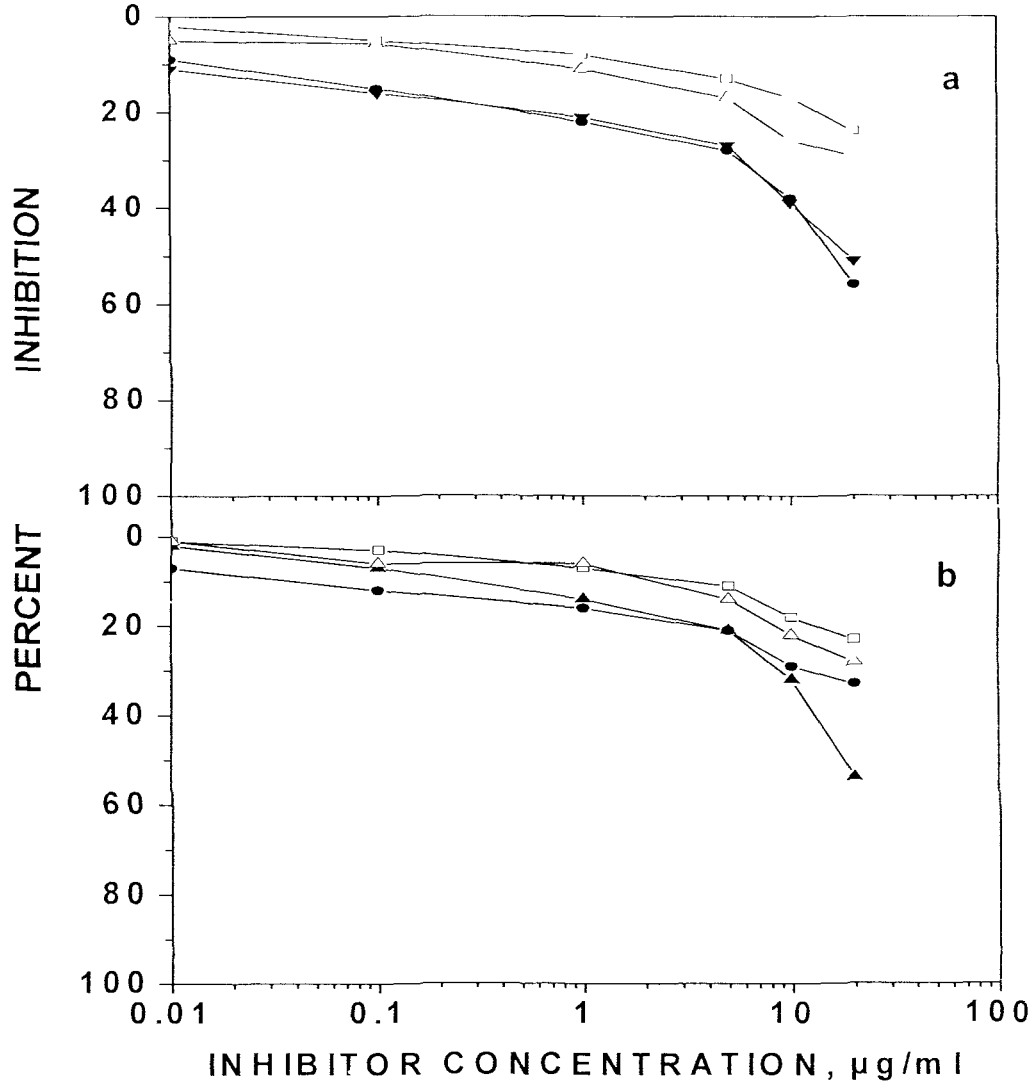


Fig. 43. Detection of autoantibodies against native and glycated HSA in the diabetic patients' sera. **(a)** Diabetic patients' sera 1 and 2 by native HSA (□, Δ), and glycated HSA (●, ▲), **(b)** diabetic patients' sera 3 and 4 by native HSA (□, Δ) and glycated HSA (●, ▲). The microtitre plates were coated with glycated HSA (20 µg/ml).

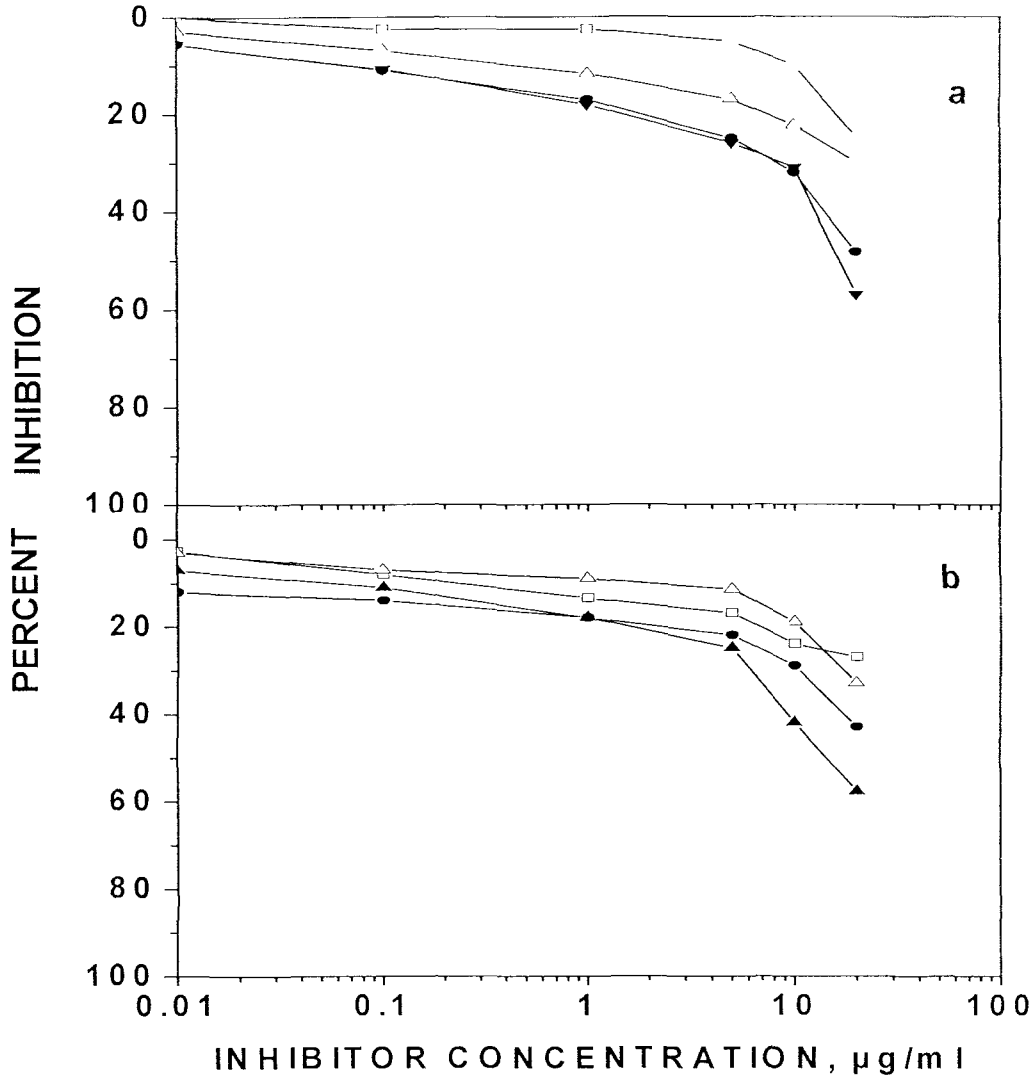


Fig. 44. Detection of autoantibodies against native and glycated HSA in the diabetic patients' sera. **(a)** Diabetic patients' sera 5 and 6 by native HSA (□, Δ), and glycated HSA (●, ▼), **(b)** diabetic patients' sera 7 and 8 by native HSA (□, Δ) and glycated HSA (●, ▲). The microtitre plates were coated with glycated HSA (20 µg/ml).

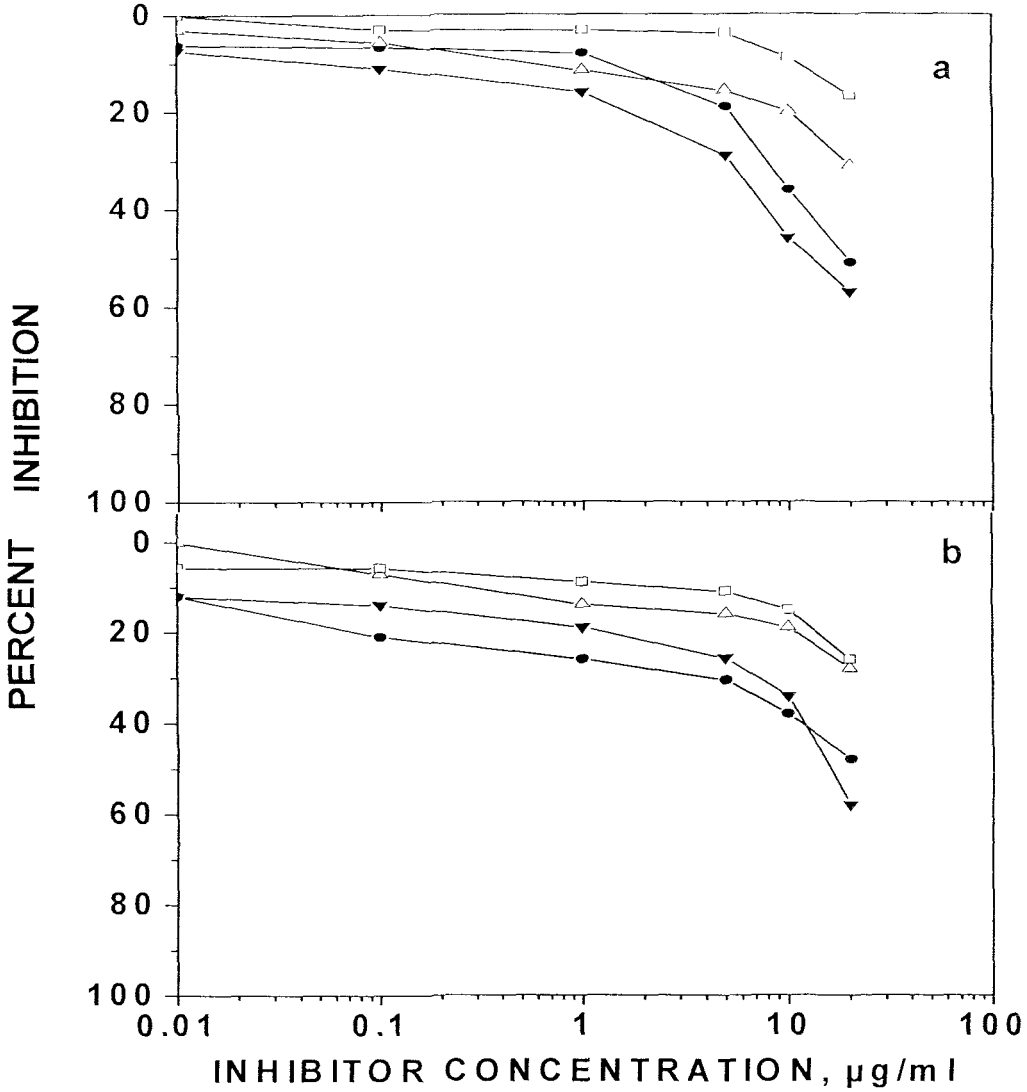


Fig. 45. Detection of autoantibodies against native and glycated HSA in the diabetic patients' sera. **(a)** Diabetic patients' sera 9 and 10 by native HSA (□, Δ), and glycated HSA (●, ▼), **(b)** diabetic patients' sera 11 and 12 by native HSA (□, Δ) and glycated HSA (●, ▼). The microtitre plates were coated with glycated HSA (20 µg/ml).

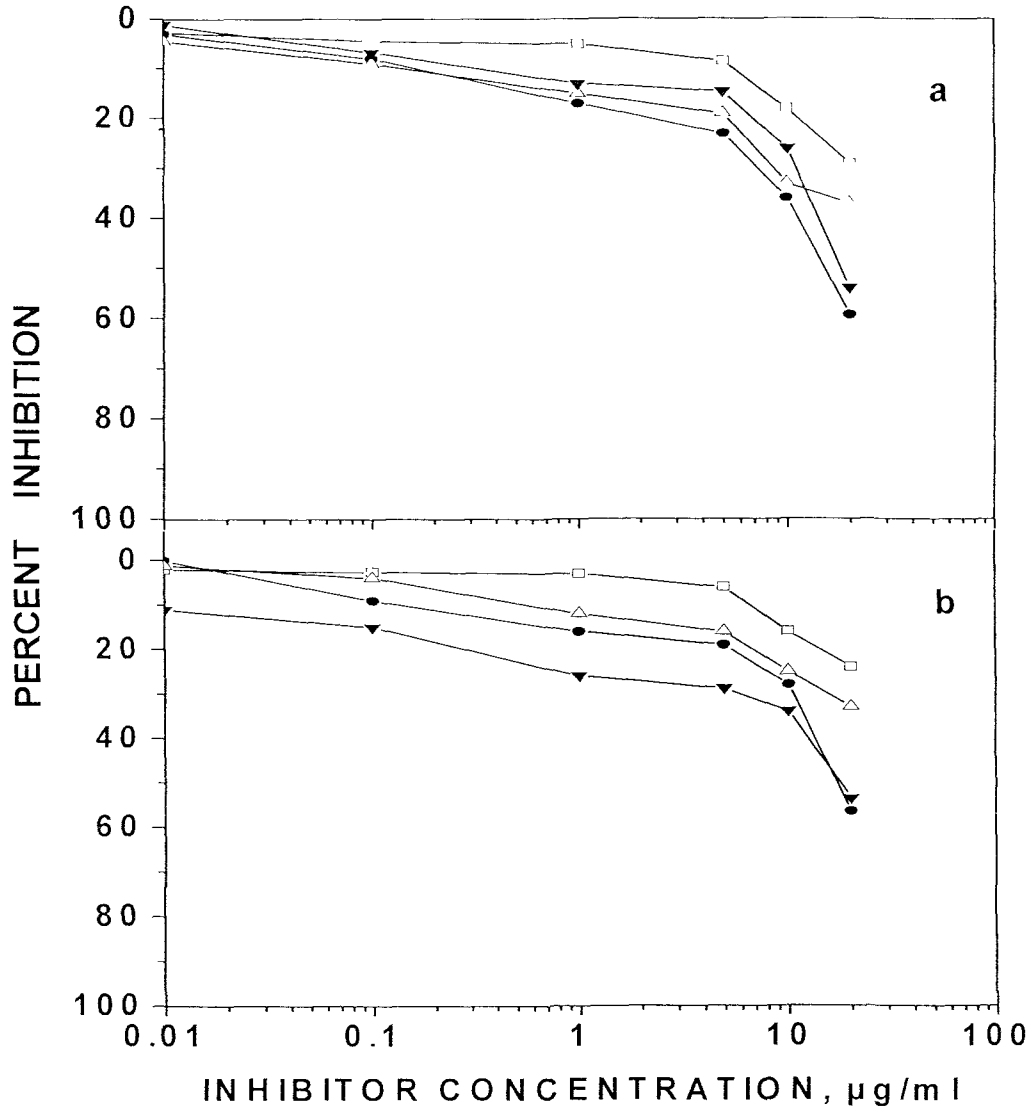


Fig. 46. Detection of autoantibodies against native and glycated HSA in the diabetic patients' sera. **(a)** Diabetic patients' sera 13 and14 by native HSA (□, Δ), and glycated HSA (●, ▼). **(b)** diabetic patients' sera 15 and 16 by native HSA (□, Δ) and glycated HSA (●, ▼). The microtitre plates were coated with glycated HSA (20 µg/ml).

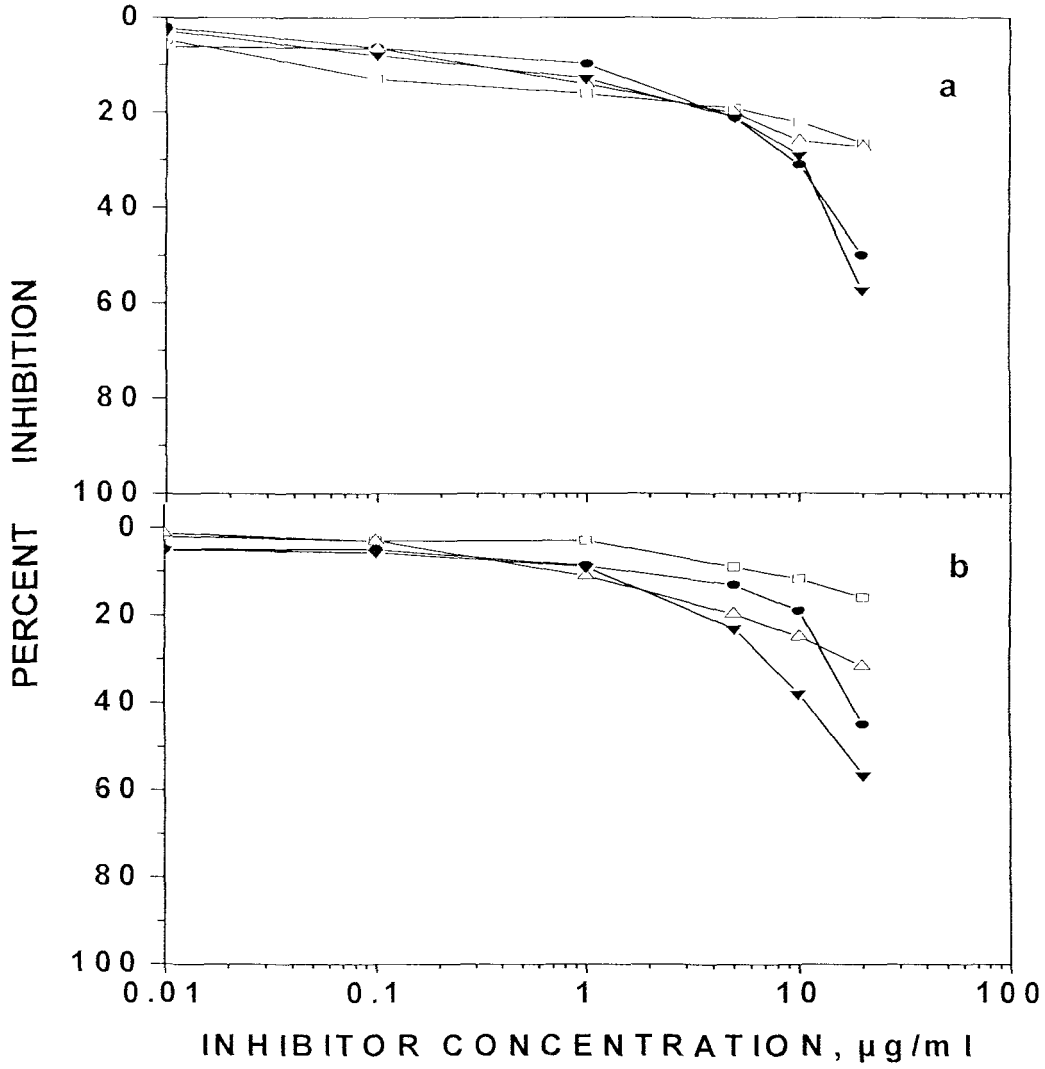


Fig. 47. Detection of autoantibodies against native and glycated HSA in the diabetic patients' sera. **(a)** Diabetic patients' sera 17 and 18 by native HSA (□, Δ), and glycated HSA (●, ▼), **(b)** diabetic patients' sera 19 and 20 by native HSA (□, Δ) and glycated HSA (●, ▼). The microtitre plates were coated with glycated HSA (20 µg/ml).

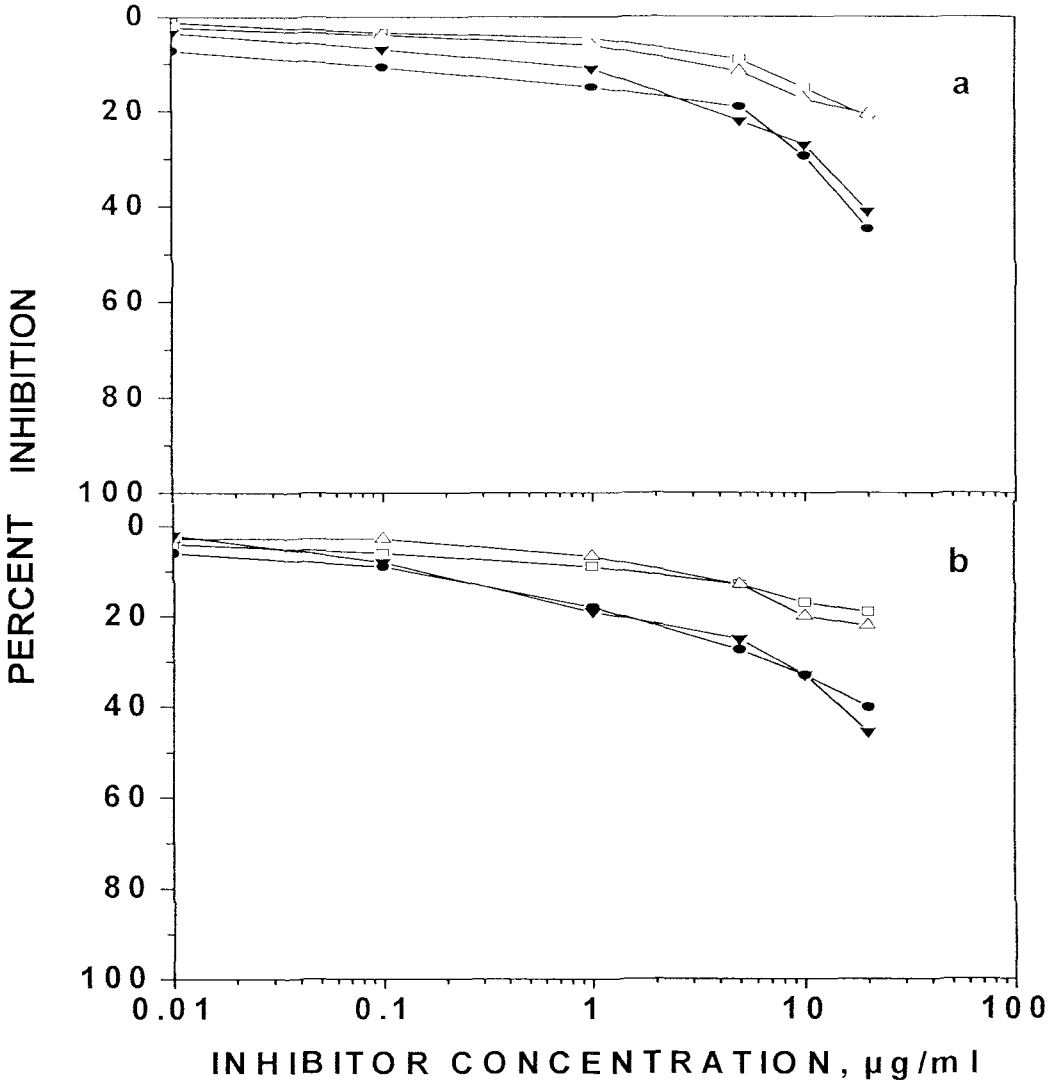


Fig. 48. Detection of autoantibodies against native and glycated HSA in the diabetic patients' sera. **(a)** Diabetic patients' sera 21 and 22 by native HSA (□, Δ), and glycated HSA (●, ▼), **(b)** diabetic patients' sera 23 and 24 by native HSA (□, Δ) and glycated HSA (●, ▼). The microtitre plates were coated with glycated HSA (20 µg/ml).

TABLE 6

Antibodies against native HSA and glycated HSA in diabetic patients' sera

	Maximum percent inhibition at 20 µg/ml	
Sera No.	Native HSA	Glycated HSA
1	29.0	56.0
2	24.0	51.0
3	23.0	32.9
4	28.0	54.0
5	25.0	48.3
6	30.0	57.0
7	27.0	43.0
8	33.0	57.8
9	16.6	51.0
10	31.0	57.0
11	28.0	48.0
12	26.0	58.0
13	29.0	59.3
14	37.0	53.9
15	24 0	56.4
16	33.0	53.4
17	26.6	49.9
18	27.3	57.3
19	16.0	45.0
20	31.7	56.7
21	21.0	44.7
22	20.6	41.0
23	19.0	40.1
24	22.0	45.6
Mean ± SD	26.2 ± 5.4	50.7 ± 7.0
(%CV)	(20.6)	(13.8)

ELISA plates were coated individually with native and glycated HSA (20 µg/ml) Significant binding with glycated HSA ($p<0.001$) than the native HSA in diabetic sera. Values in paranthesis indicate percent of coefficient of variation.

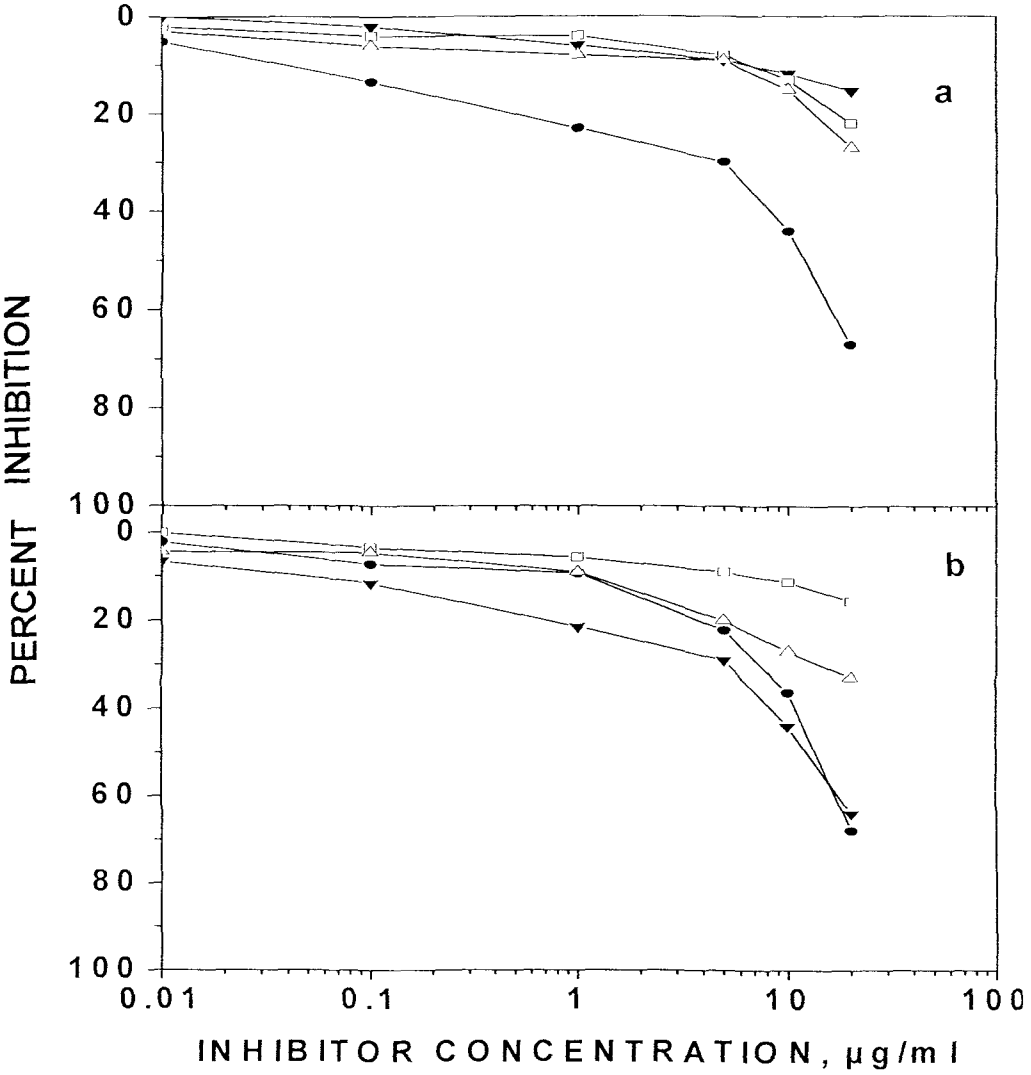


Fig. 49. Detection of autoantibodies against native and glycated HSA in the normal and diabetic retinopathic patients' sera. **(a)** Normal and diabetic retinopathic patients' sera by native HSA (□, Δ), and glycated HSA (●, ▼), **(b)** diabetic retinopathic patients' sera 2 and 3 by native HSA (□, Δ) and glycated HSA (●, ▼). The microtitre plates were coated with glycated HSA (20 µg/ml).

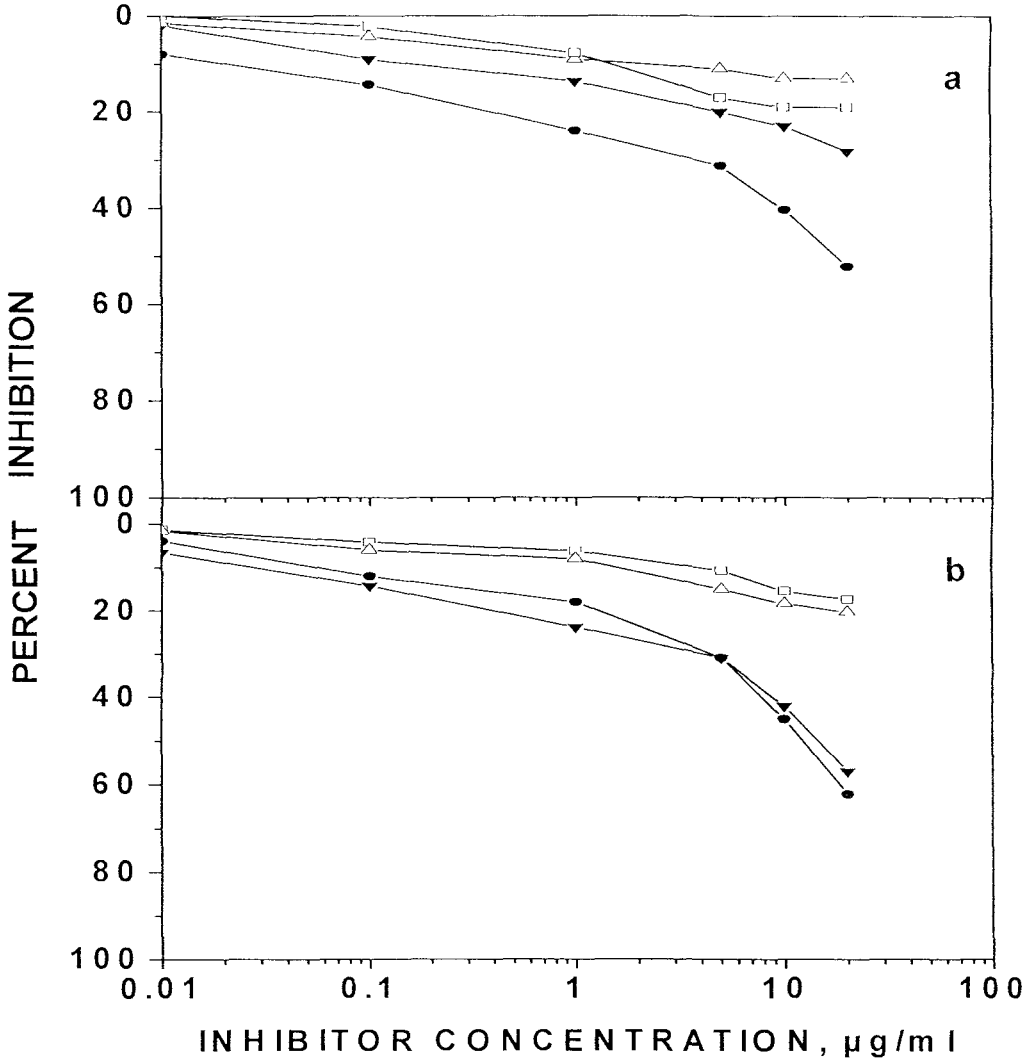


Fig. 50. Detection of autoantibodies against native and glycated HSA in the normal and diabetic nephropathic patients' sera. **(a)** Normal and diabetic nephropathic patients' sera by native HSA (□, Δ), and glycated HSA (●, ▼), **(b)** diabetic nephropathic patients'sera 2 and 3 by native HSA (□, Δ) and glycated HSA (●, ▼). The microtitre plates were coated with glycated HSA (20 µg/ml).

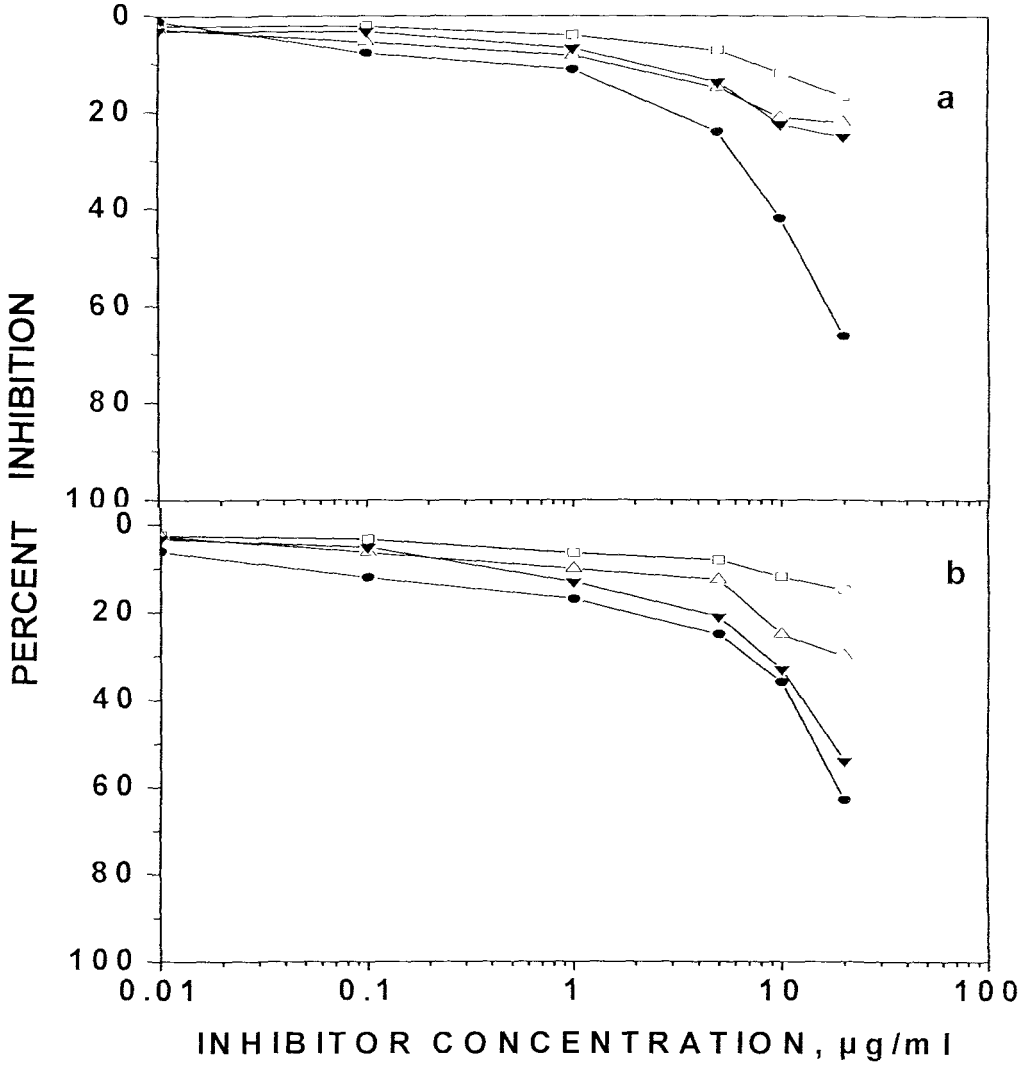


Fig. 51. Detection of autoantibodies against native and glycated HSA in the normal and diabetic artherosclerotic patients' sera. **(a)** Normal and diabetic artherosclerotic subjects' sera by native HSA (□, Δ), and glycated HSA (●, ▼), **(b)** diabetic artherosclerotic patients' sera 2 and 3 by native HSA (□, Δ) and glycated HSA (●, ▼). The microtitre plates were coated with glycated HSA (20 µg/ml).

TABLE 7

Antibodies against native HSA and glycated HSA in diabetic patients' sera with secondary complications

Sera Types	Maximum percent inhibition at 20 µg/ml Native HSA	Glycated HSA
Diabetic Retinopathy		
1	15.3	65.2
2	16.0	68.0
3	33.0	64.0
Diabetic Nephropathy		
1	28.2	52.1
2	17.3	62.3
3	20.2	57.0
Diabetic Artherosclerosis		
1	16.7	66.2
2	14.9	63.0
3	30.0	54.1
Normal Subjects		
1	22.0	27.0
2	13.0	19.0
3	22.0	25.0

ELISA plates were coated with native and glycated HSA at 20 µg/ml
Statistically significant binding of glycated HSA ($p<0.001$) than native HSA in diabetic sera. Normal individuals showed negligible binding with either of the antigen.

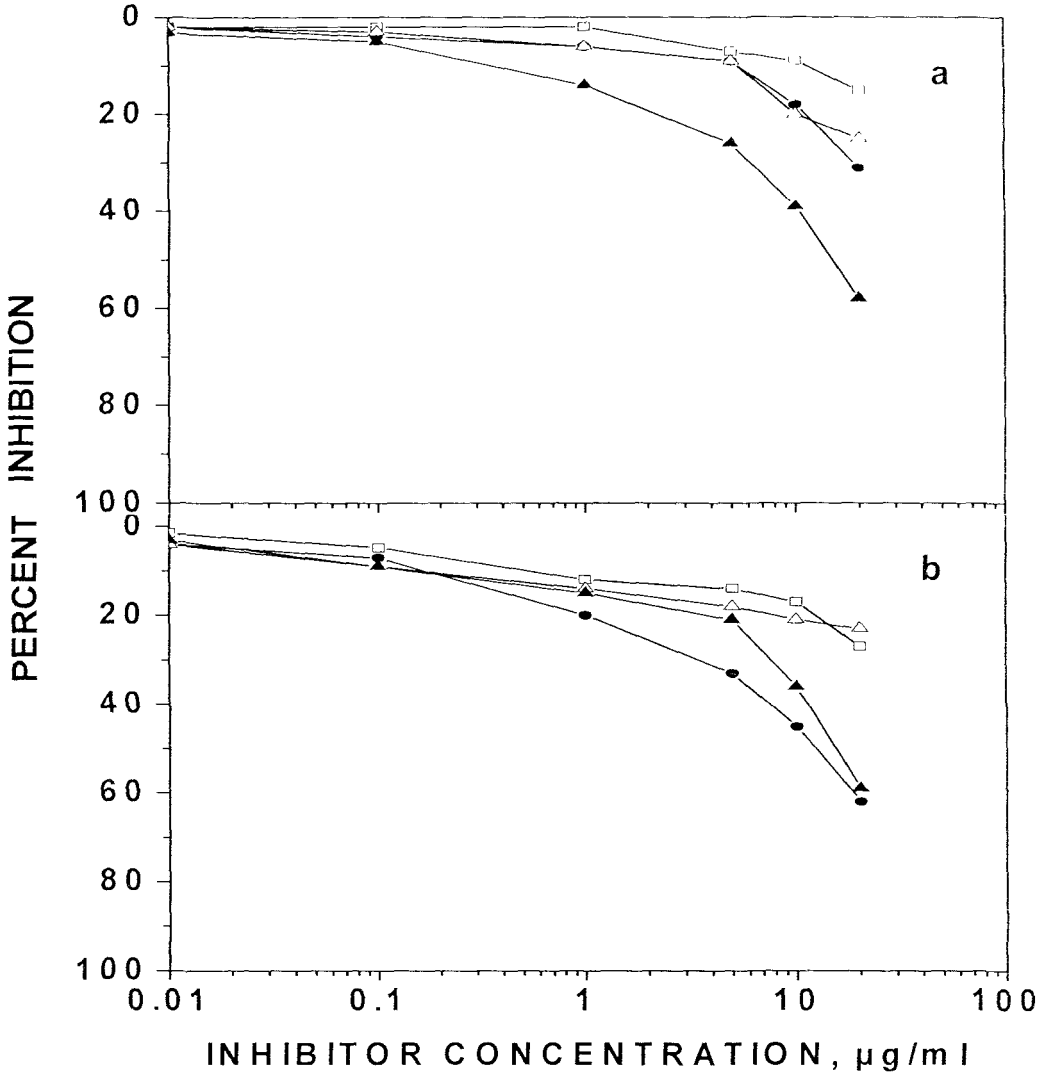


Fig. 52. Detection of autoantibodies against native and ROS-glycated HSA in the diabetic patients' sera. (a) Diabetic patients' sera 1 and 2 by native HSA (□, Δ), and ROS-glycated HSA (●, ▲), (b) diabetic patients' sera 3 and 4 by native HSA (□, Δ) and glycated HSA (●, ▲). The microtitre plates were coated with ROS-glycated HSA (20 µg/ml).

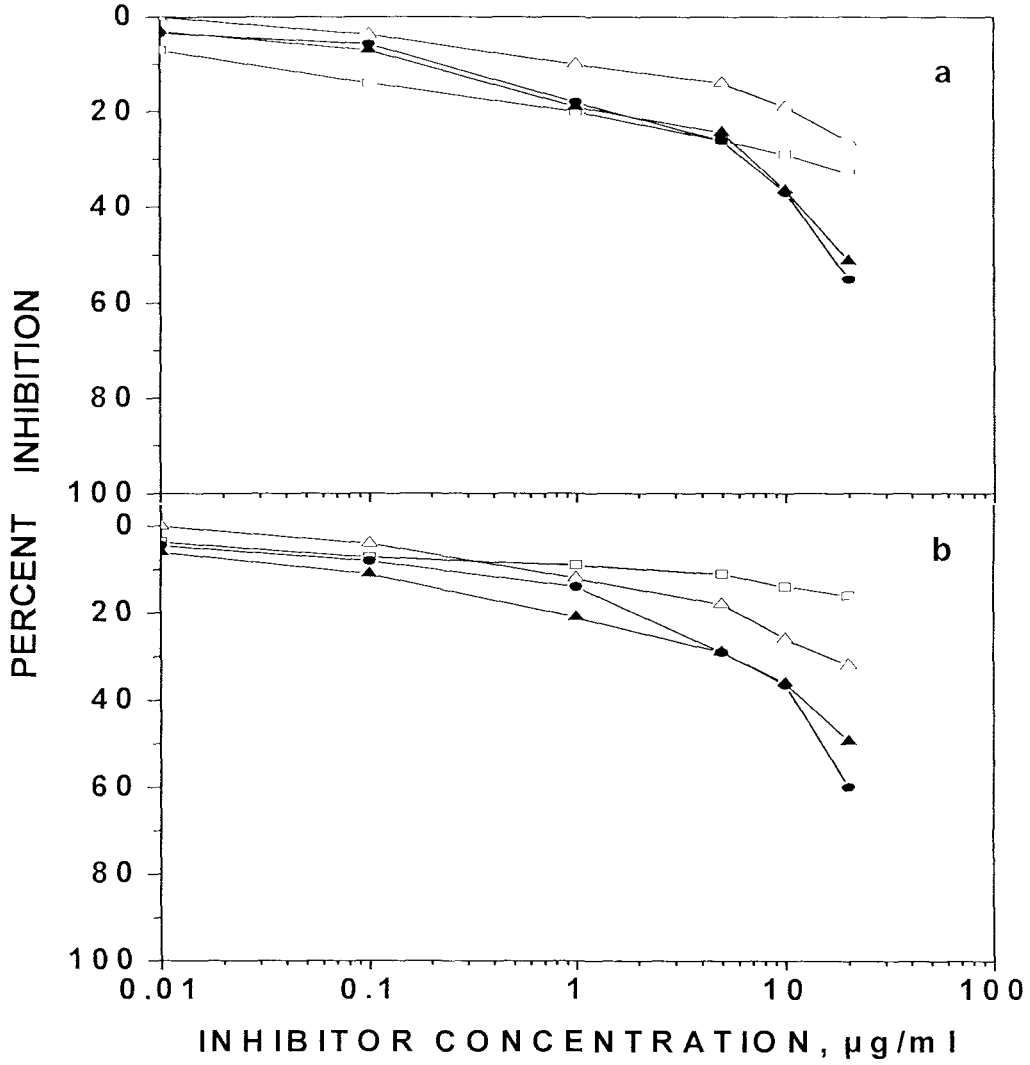


Fig. 53. Detection of autoantibodies against native and ROS-glycated HSA in the diabetic patients' sera. **(a)** Diabetic patients' sera 5 and 6 by native HSA (□, Δ), and ROS-glycated HSA (●, ▲), **(b)** diabetic patients' sera 7 and 8 by native HSA (□, Δ) and glycated HSA (●, ▲). The microtitre plates were coated with ROS-glycated HSA (20 µg/ml).

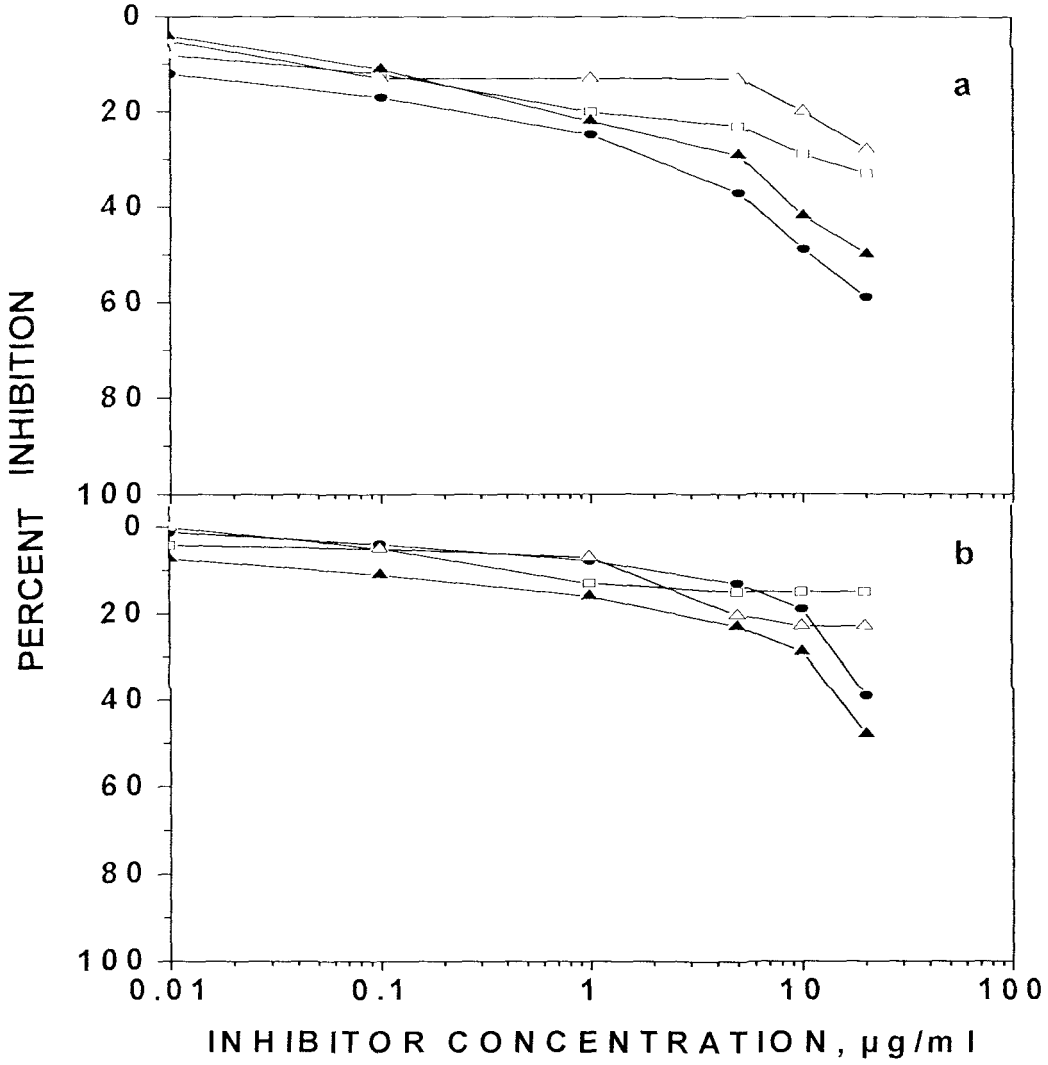


Fig. 54. Detection of autoantibodies against native and ROS-glycated HSA in the diabetic patients' sera. **(a)** Diabetic patients' sera 9 and 10 by native HSA (□, Δ), and ROS-glycated HSA (●, ▲), **(b)** diabetic patients' sera 11 and 12 by native HSA (□, Δ) and glycated HSA (●, ▲). The microtitre plates were coated with ROS-glycated HSA (20 µg/ml).

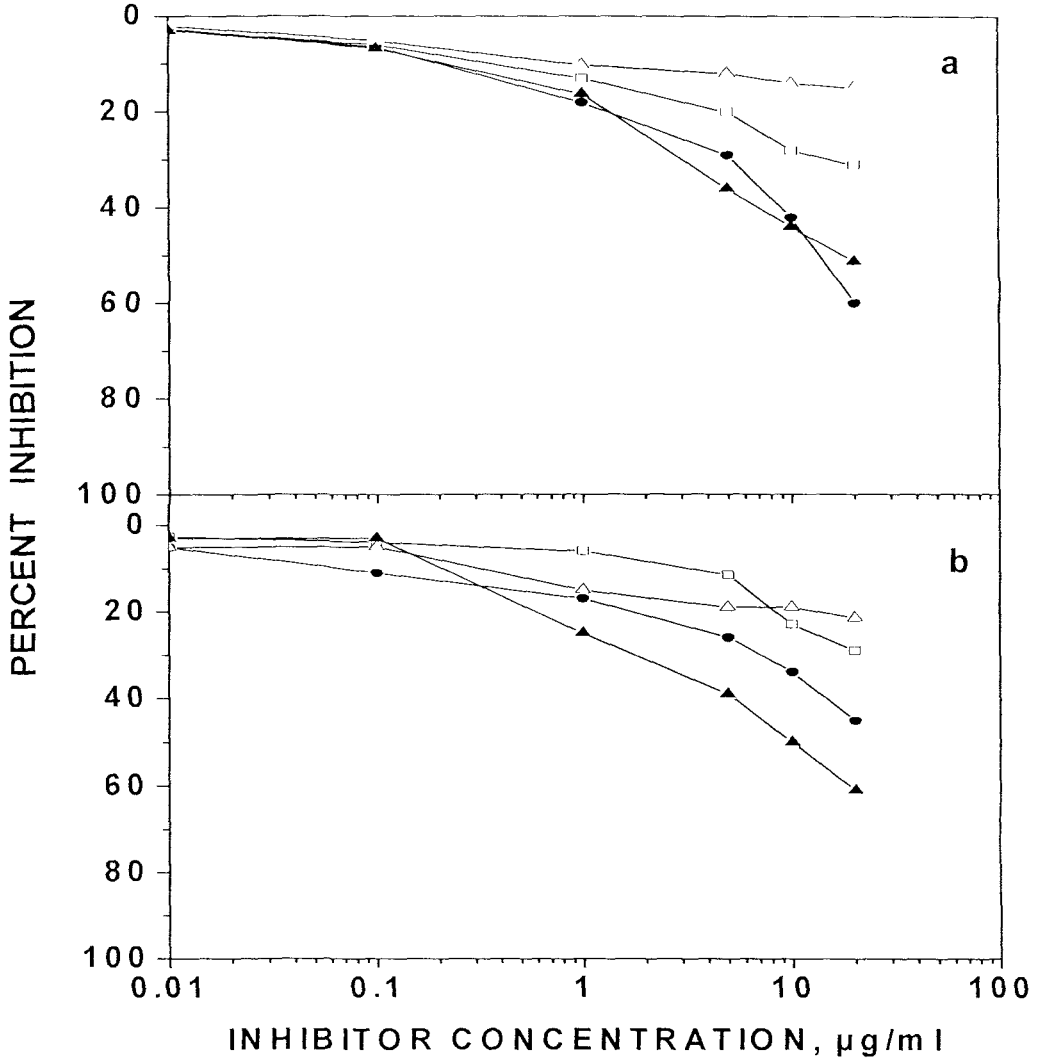


Fig. 55. Detection of autoantibodies against native and ROS-glycated HSA in the diabetic patients' sera. **(a)** Diabetic patients' sera 13 and 14 by native HSA (□, Δ), and ROS-glycated HSA (●, ▲), **(b)** diabetic patients' sera 15 and 16 by native HSA (□, Δ) and glycated HSA (●, ▲). The microtitre plates were coated with ROS-glycated HSA (20 µg/ml).

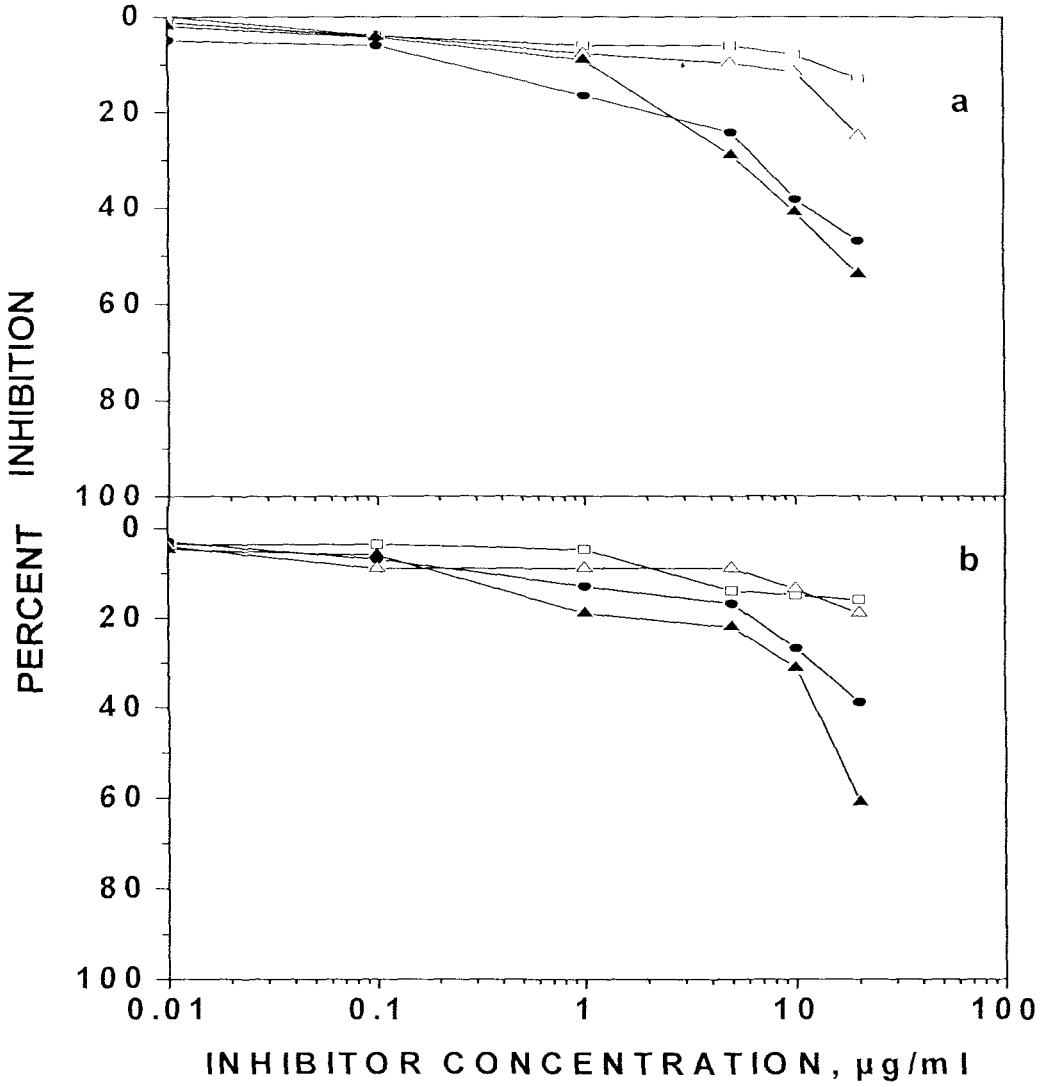


Fig. 56. Detection of autoantibodies against native and ROS-glycated HSA in the diabetic patients' sera. **(a)** Diabetic patients' sera 17 and 18 by native HSA (□, Δ), and ROS-glycated HSA (●, ▲), **(b)** diabetic patients' sera 19 and 20 by native HSA (□, Δ) and glycated HSA (●, ▲). The microtitre plates were coated with ROS-glycated HSA (20 µg/ml).

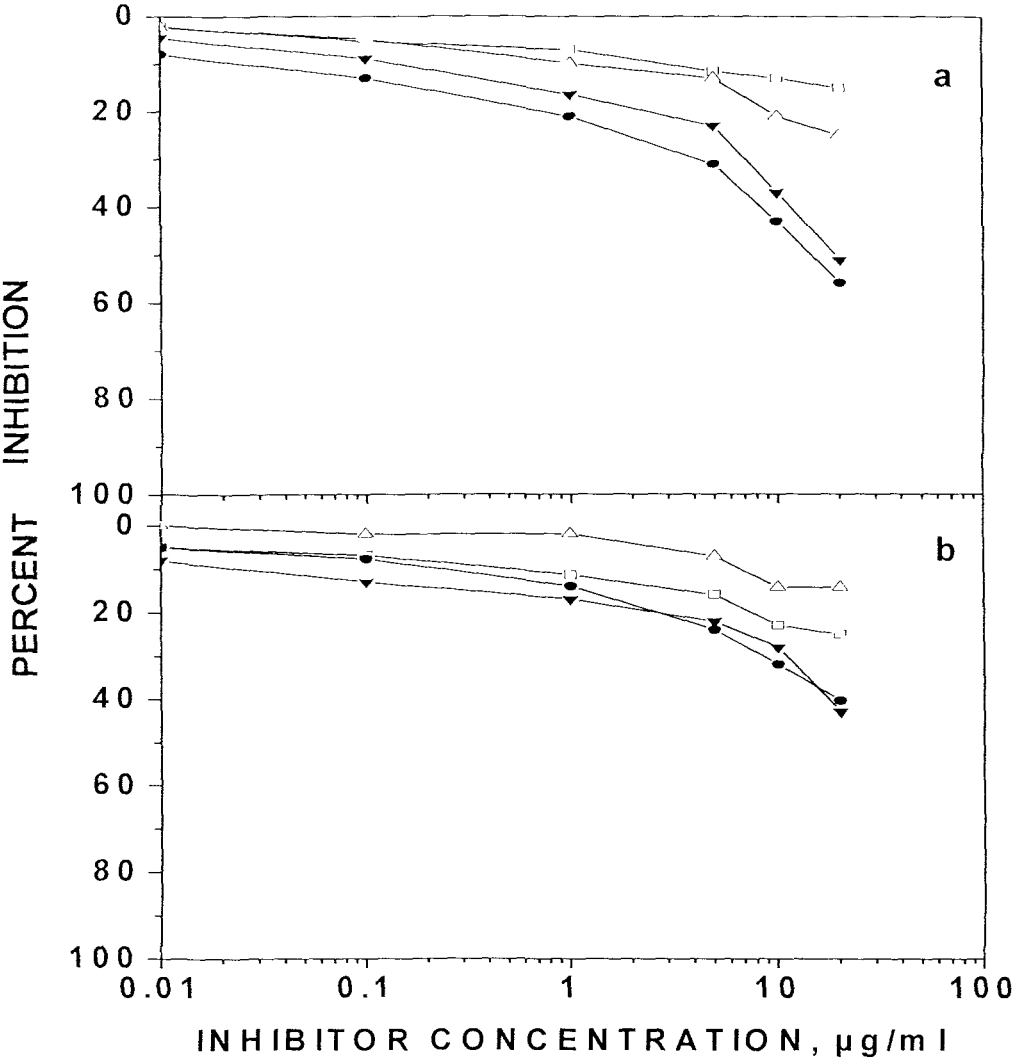


Fig. 57. Detection of autoantibodies against native and ROS-glycated HSA in the diabetic patients' sera. **(a)** Diabetic patients' sera 21 and 22 by native HSA (□, Δ), and ROS-glycated HSA (●, ▲), **(b)** diabetic patients' sera 23 and 24 by native HSA (□, Δ) and glycated HSA (●, ▲). The microtitre plates were coated with ROS-glycated HSA (20 µg/ml).

TABLE 8

Antibodies against native HSA and ROS-glycated HSA in diabetic patients' sera

Sera No.	Maximum percent inhibition at 20 µg/ml	
	Native HSA	ROS-Glycated HSA
1	15.0	31.0
2	25.0	58.0
3	27.0	62.0
4	23.0	59.0
5	33.1	55.1
6	26.3	51.3
7	16.3	60.0
8	31.0	49.5
9	32. 5	58.6
10	28.0	50.0
11	15.0	39.0
12	23.0	48.5
13	31.0	60.0
14	15.3	52.0
15	29.0	45.0
16	21.4	61.0
17	13.0	47.0
18	25.0	54.0
19	16.0	38.9
20	19.0	61.0
21	15.3	54.0
22	24.0	51.0
23	25.2	40.4
24	14.3	43.0
Mean ± SD	22.6 ± 6.4	51.6 ± 8.5
(%CV)	(28.3)	(16.5)

ELISA plates were coated with native and ROS-glycated HSA at 20 µg/ml
Significant binding with ROS-glycated HSA ($p<0.001$) than the native HSA in diabetic sera. Values in paranthesis indicate percent of coefficient of variation.

The specific binding of the autoantibodies was remarkably higher for ROS-glycated HSA ($p < 0.001$) than native HSA in the sera of diabetic patients having secondary complications (Figs. 58-60). In the case of nine sera, the observed maximum inhibition with native and ROS-glycated HSA was in the range of 16% to 37% and 32.9% to 59.3%, respectively. The inhibition data of native HSA and ROS-glycated HSA with diabetic patients' sera with secondary complications are summarized in Table 9.

Purification of diabetic patient's IgG

Diabetic retinopathic patient's IgG (serum 2) purified by affinity chromatography on Protein A-Agarose column eluted as a symmetrical single peak (Fig. 61). Purified IgG migrated as a single homogenous band on SDS-PAGE under non-reducing conditions (Fig. 61, inset).

Band Shift Assay

The binding of native and modified HSA to diabetic retinopathic patient's IgG (serum 2) was detected by band shift assay. Constant amount of antigens were incubated with varying amount of diabetic patient IgG for 2 hr at 37°C and overnight at 4°C. Immune complexes were electrophoresed on 5.5% polyacrylamide gel for 3 hr at 50 mV. Figure 62 showed the binding of diabetic patient IgG to glycated HSA and Figure 63 showed the binding of diabetic patients IgG to ROS-glycated HSA. With increasing concentration of IgG, the formation of high molecular weight immune complexes increased as judged by increase in intensity of immune complexs, whereas, the amount of unbound HSA showed a proportional decrease in its band intensity.

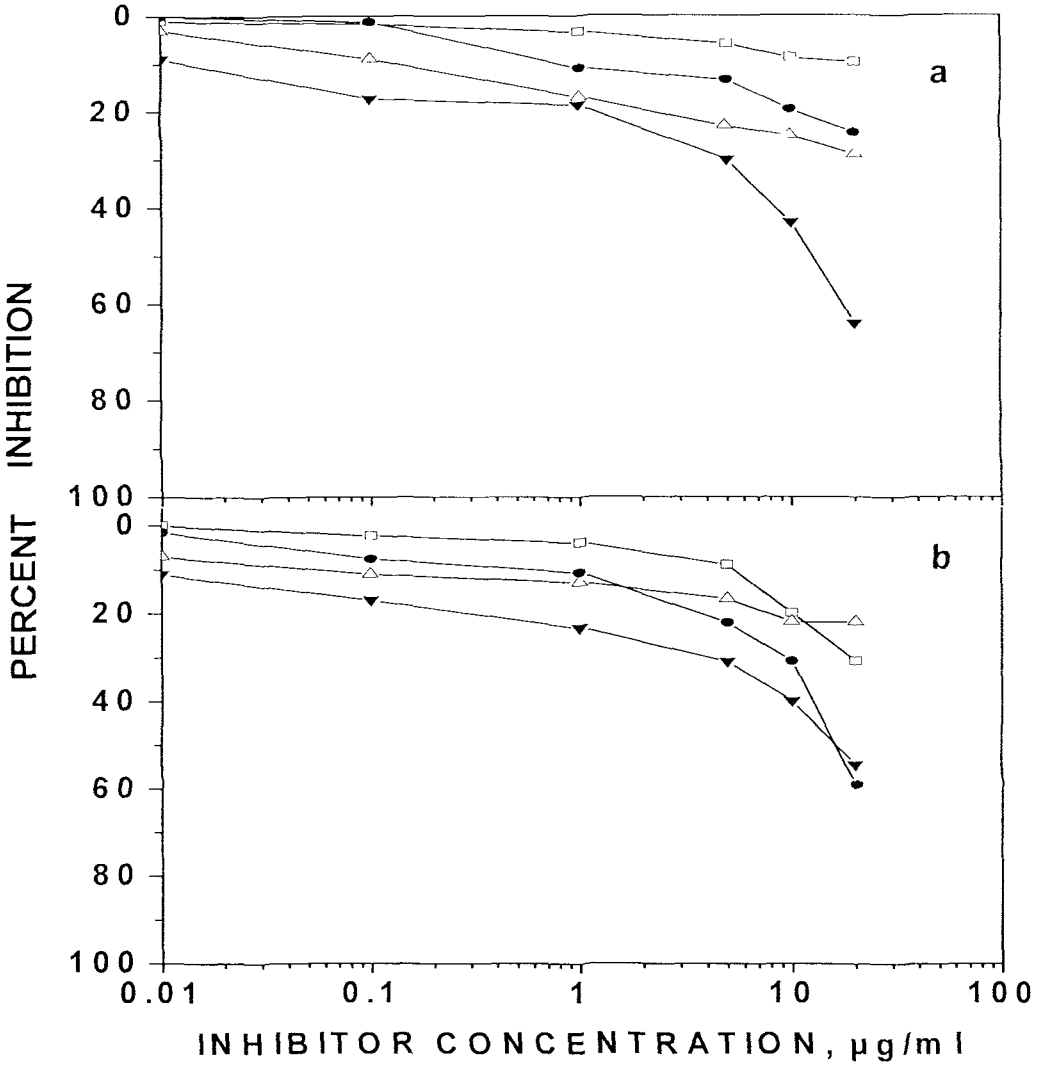


Fig. 58. Detection of autoantibodies against native and ROS-glycated HSA in the normal and diabetic retinopathic patients' sera. **(a)** Normal and diabetic retinopathic subjects' sera by native HSA (□, Δ), and ROS-glycated HSA (●, ▼), **(b)** diabetic retinopathic patients' sera 2 and 3 by native HSA (□, Δ) and glycated HSA (●, ▼). The microtitre plates were coated with ROS-glycated HSA (20 µg/ml).

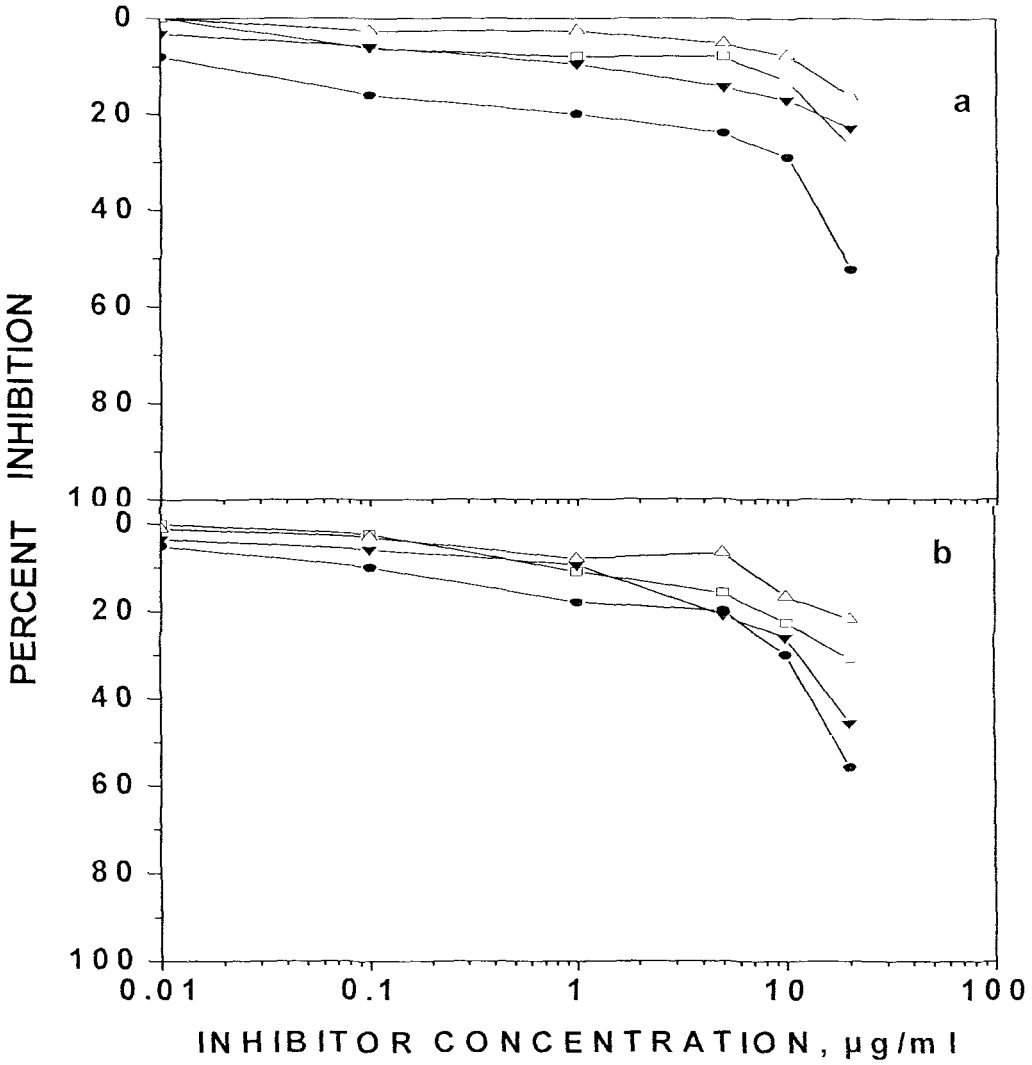


Fig. 59. Detection of autoantibodies against native and ROS-glycated HSA in the normal and diabetic nephropathic patients' sera. **(a)** Normal and diabetic nephropathic patients' sera by native HSA (□, Δ), and ROS-glycated HSA (●, ▼), **(b)** diabetic nephropathic patients' sera 2 and 3 by native HSA (□, Δ) and glycated HSA (●, ▼). The microtitre plates were coated with ROS-glycated HSA (20 µg/ml).

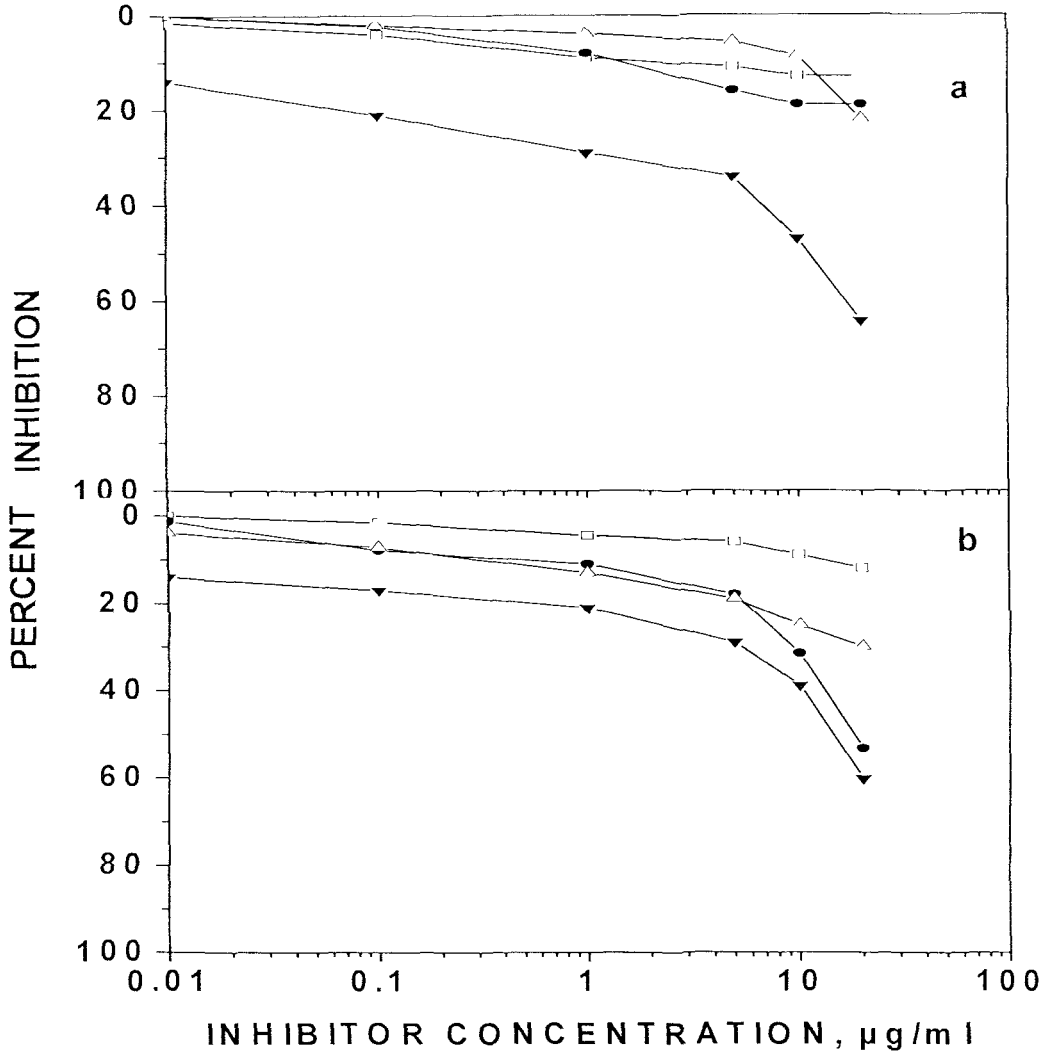


Fig. 60. Detection of autoantibodies against native and ROS-glycated HSA in the normal and diabetic artherosclerotic patients' sera. **(a)** Normal and diabetic artherosclerotic subjects' sera by native HSA (□, Δ), and ROS-glycated HSA (●, ▼), **(b)** diabetic artherosclerotic patients' sera 2 and 3 by native HSA (□, Δ) and glycated HSA (●, ▼). The microtitre plates were coated with ROS-glycated HSA (20 µg/ml).

TABLE 9

Antibodies against native HSA and ROS-glycated HSA in diabetic patients' sera with secondary complications

Sera Types	Maximum percent inhibition at 20 µg/ml	
	Native HSA	ROS-Glycated HSA
Diabetic Retinopathy		
1	29.0	64.3
2	30.9	59.1
3	33.0	54.5
Diabetic Nephropathy		
1	27.0	52.4
2	16.0	50.0
3	31.0	56.0
Diabetic Artherosclerosis		
1	22.0	64.0
2	14.9	53.4
3	30.0	60.0
Normal Subjects		
1	22.0	24.4
2	16.4	23.0
3	13.0	19.0

ELISA plates were coated with native and ROS-glycated HSA at 20 µg/ml Statistically significant binding of ROS-glycated HSA ($p<0.001$) than native HSA in diabetic sera. Normal individuals showed negligible binding with either of the antigen.

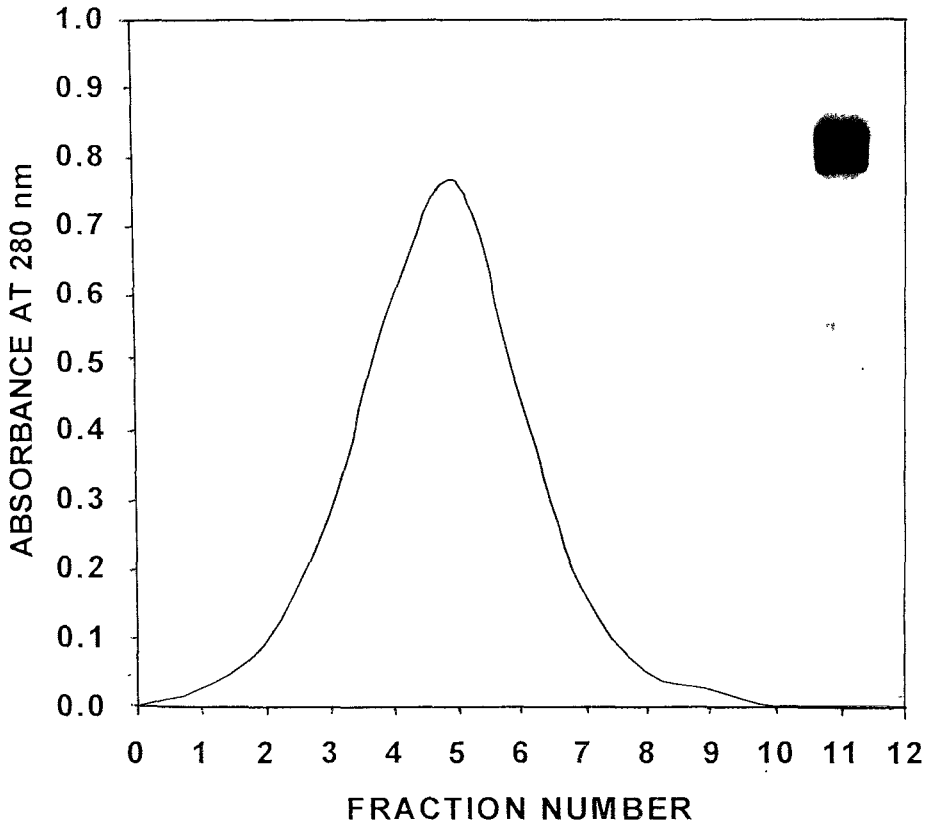


Fig. 61. Elution profile of diabetic retinopathic patient IgG (serum 2) on Protein-A Agarose affinity column. **Inset:** SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.

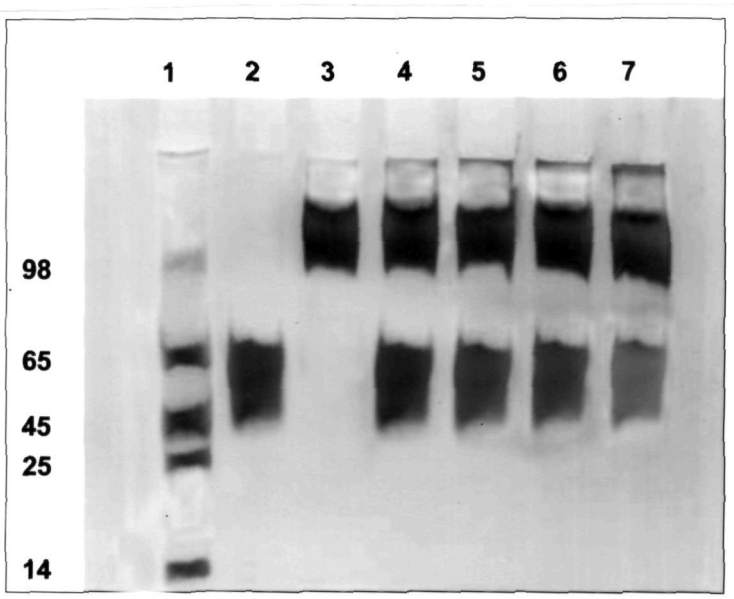


Fig. 62. Band shift assay of diabetic retinopathic patient's IgG (serum 2) binding to glycated HSA. Glycated HSA (10 μg) (lane 2) and diabetic patient's IgG (10 μg) (lane 3) were incubated with buffer alone. Increasing amounts of patient IgG (10, 20, 30 and 40 μg) with constant amount of glycated HSA (10 μg) through lanes 4 to 7. respectively were incubated for 2 hr at 37°C and overnight at 4°C. Lane 1 contain protein molecular weight marker (98–14 kDa). Electrophoresis was carried out on 5.5% polyacrylamide gel for 3 hr at 50 V.

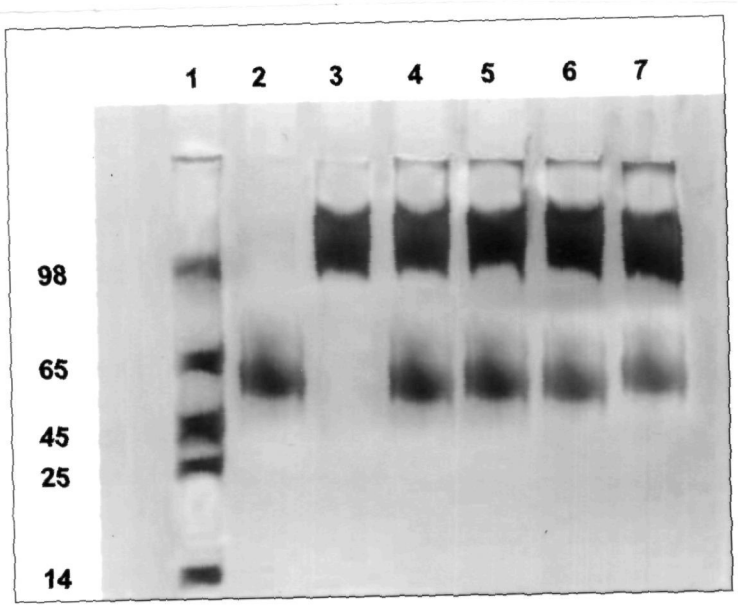


Fig. 63. Band shift assay of diabetic retinopathic patient's IgG (serum 2) binding to ROS-glycated HSA. ROS-glycated HSA (10 μg) (lane 2) and diabetic patient's IgG (10 μg) (lane 3) were incubated with buffer alone. Increasing amount of patient IgG (10, 20, 30 and 40 μg) with constant amount of ROS-glycated HSA (10 μg) through lanes 4 to 7, respectively were incubated for 2 hr at 37°C and overnight at 4°C. Lane 1 contain protein molecular weight marker (98–14 kDa). Electrophoresis was carried out on 5.5% polyacrylamide gel for 3 hr at 50 V.

Probing glycation in diabetic patients using anti-glycated HSA antibody

Albumin and immunoglobulin-G (IgG) was isolated from sera of diabetic patients showing maximum recognition to both modified HSA (diabetic retinopathic patients' sera 1-3) (Figs. 64 and 65, respectively). IgG and albumin was also isolated from normal human serum (NHS). The purity of IgGs was ascertained by a single homogenous band on the 7.5% polyacrylamide gel (Fig 64, inset). The purity and concentration of the HSA preparations were ascertained by elution profile compared with the commercially available HSA elution profile (Fig 65), showing that the isolated protein is indeed serum albumin.

Anti-glycated HSA antibody was used as a probe to detect effect of glycation in diabetic patient's serum proteins. Serum albumin isolated from normal individuals served as control. Immune complexes were made between isolated serum albumin and anti-glycated HSA antibody. Microtitre plates were coated with glycated HSA at the concentration of 20 μg/ml. Isolated serum albumin from three diabetic patients showed high recognition to anti-glycated HSA antibody, showing inhibition ranging from 51.7% to 62.9% (Fig. 66a). Samples of HSA from NHS showed inhibition of 27% (Fig. 66a).

Same diabetic patients and normal individual sera have been used to isolate IgG. Similarly, the immune complexes were formed between isolated IgGs and anti-glycated HSA antibody. The range of inhibition of anti-glycated HSA antibody at 20 μg/ml with diabetic patient IgG were found to be 47.4% to 62.9%. (Fig. 66b). IgG isolated from NHS gave negligible inhibition of 19% at the same concentration (Fig. 66b).

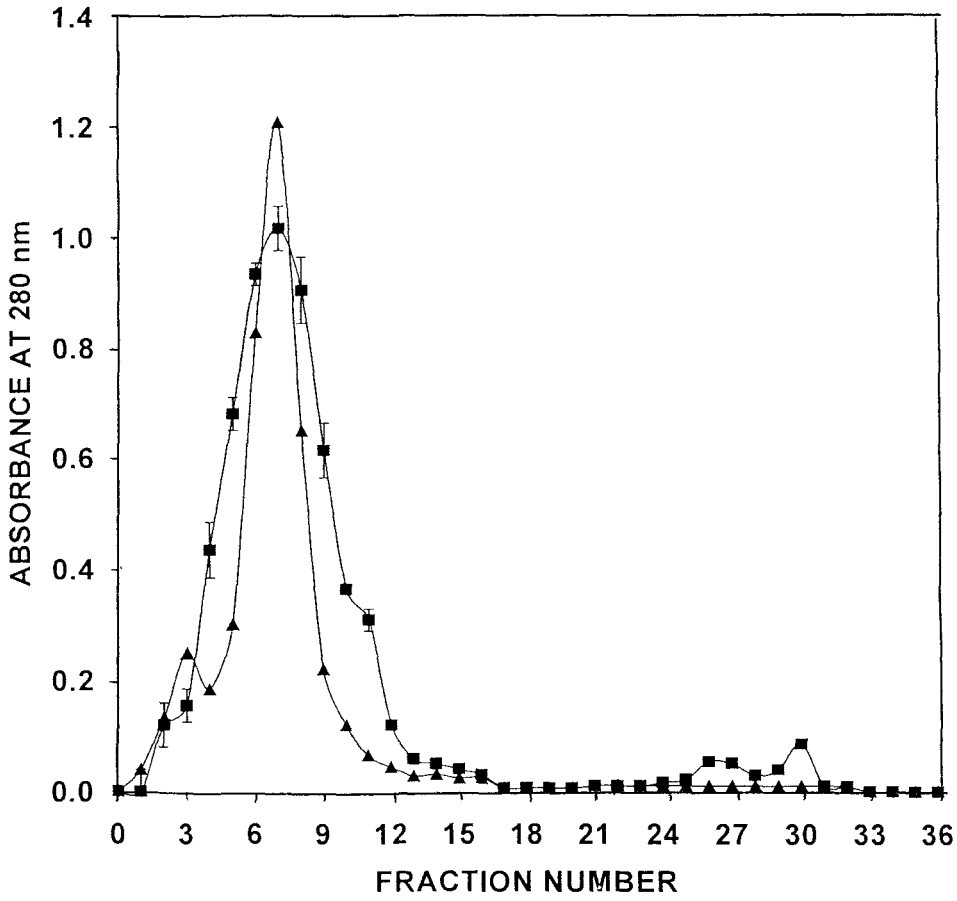


Fig. 64. Gel filtration column chromatography of commercially available HSA (—▲—) and serum isolated albumin (—■—) using Sephacryl^{TM} S–200. Each point represents mean ± SD of four values.

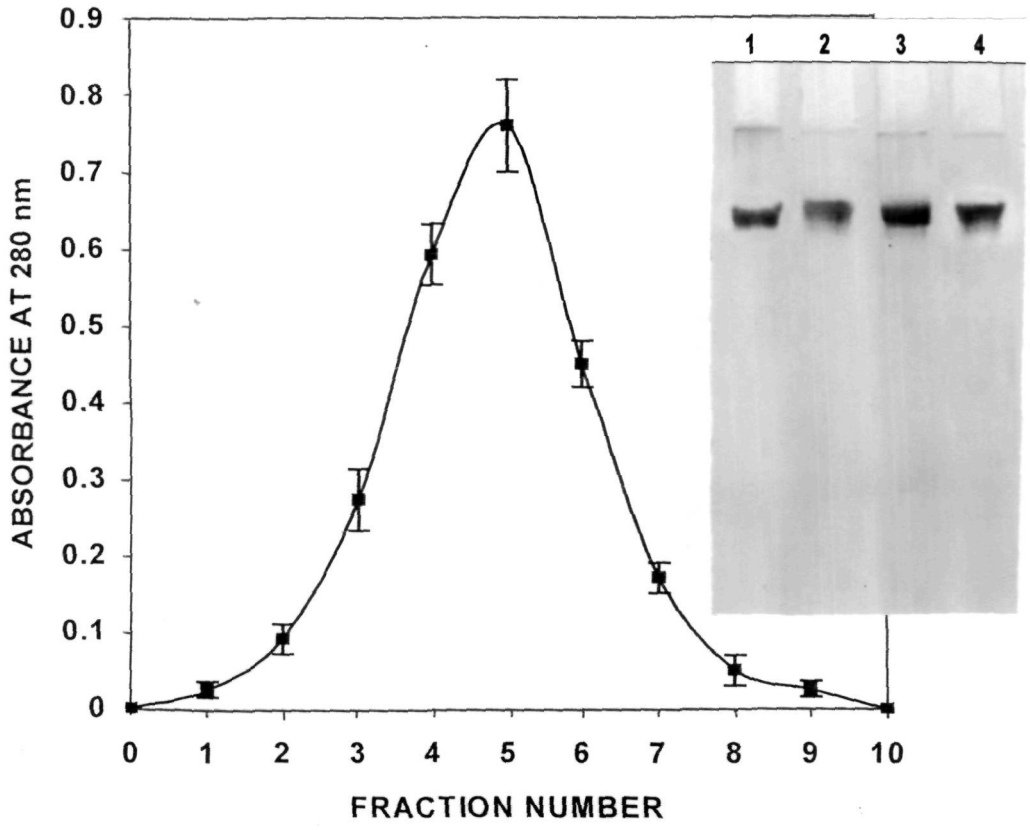


Fig. 65. Elution profile of isolated IgG on Protein A-Agarose affinity column. **Inset:** SDS-PAGE of purified IgGs on 7.5% polyacrylamide gel. Each point represents mean ± SD of four values.

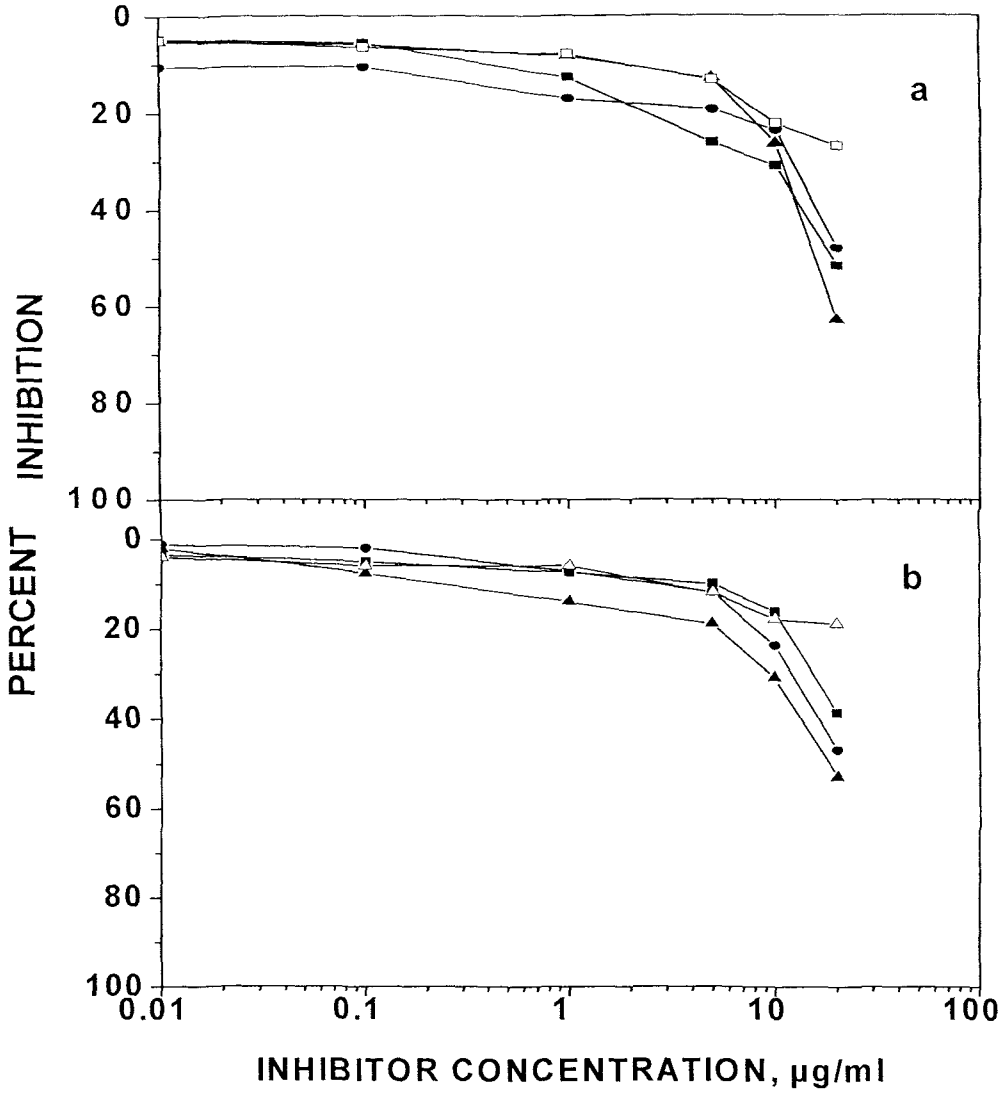


Fig. 66. Inhibition of anti-glycated IgG binding to serum isolated proteins. Microtitre plates were coated with glycated HSA (20 µg/ml). The competitors were **(a)** HSA isolated from normal human serum (□) and 3 diabetic patients (■, ●, ▲), **(b)** IgG isolated from normal human sera (Δ) and 3 diabetic patients (■, ●, ▲).

Probing ROS damage in diabetic patients using anti-ROS-glycated HSA antibody

Serum albumin samples isolated from normal and diabetic patients were used. The immune complexes were formed between isolated HSA and anti-ROS-glycated HSA antibody. Diabetic patients' isolated albumin samples showed inhibition of 45%, 50% and 53% at 20 μg/ml (Fig. 67a). However, albumin from NHS showed inhibition of only 22% at 20 μg/ml.

Isolate IgG from normal and diabetic subjects' was also used to form immune complex with anti-ROS-glycated HSA antibody. The range of inhibition at 20 μg/ml with anti-ROS-glycated HSA antibody was 40.3% to 49%, (Fig. 67b). no appreciable binding was observed with NHS.

The percent inhibition data of serum isolated proteins from diabetic patients' and NHS with anti-glycated and anti-ROS glycated HSA antibody are given in Table 10.

Reactivity of rheumatoid arthritic patients' sera with native and modified HSA

Sera from twelve rheumatoid arthritic patients' both sero negative and positive were tested for binding to native, glycated and ROS-glycated HSA. The binding pattern of serum antibodies to native and modified HSA samples was determined by direct binding ELISA. Nearly all the twelve rheumatoid arthritic patients' sera showed stronger binding to glycated HSA ($p < 0.001$) and ROS-glycated HSA ($p < 0.001$) (Fig. 68). No appreciable binding was observed with the sera of normal subjects. The specificity of arthritic sera for native and both modified HSA was evaluated by inhibition ELISA (Fig. 69-71).

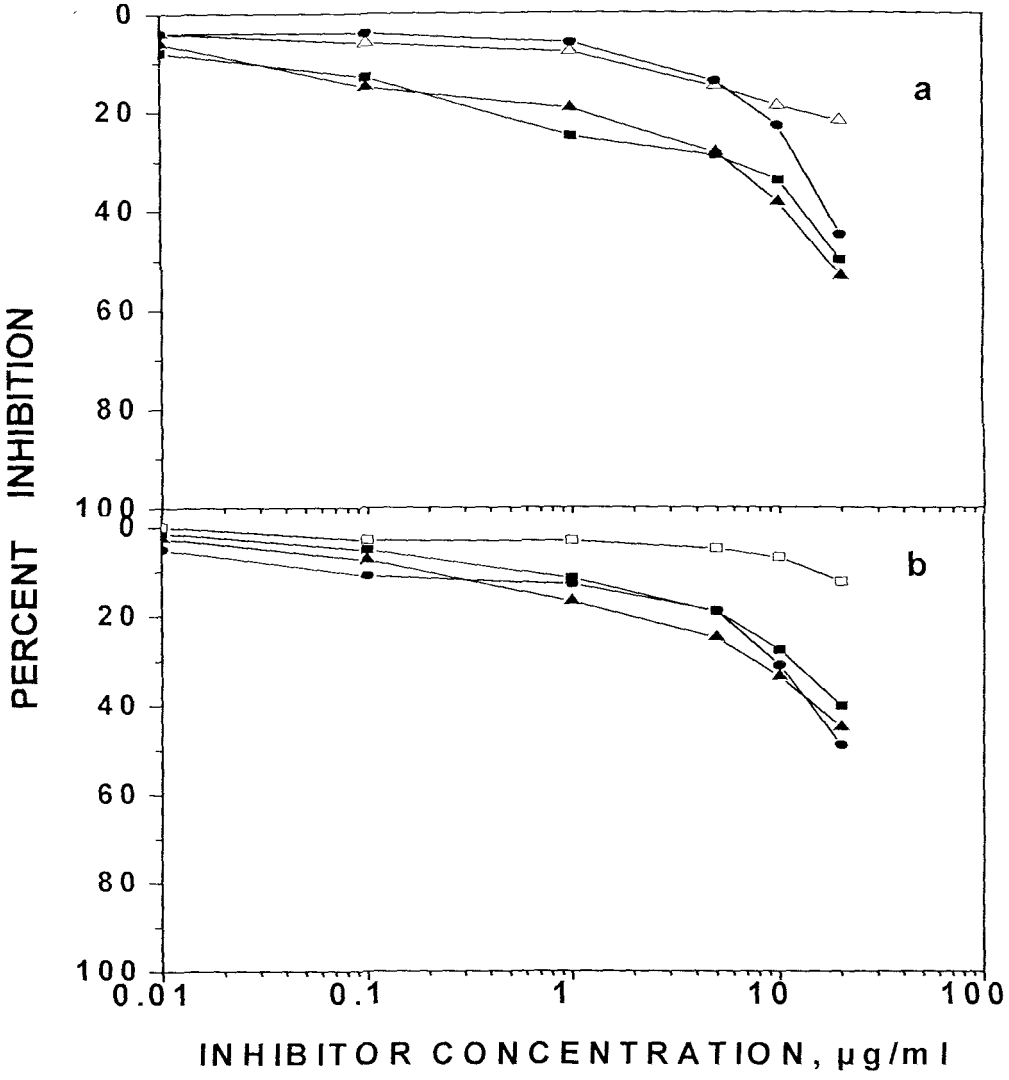


Fig. 67. Inhibition of anti-ROS-glycated IgG binding to serum isolated proteins. Microtitre plates were coated with ROS-glycated HSA (20 µg/ml). The competitors were **(a)** HSA isolated from one normal human serum (Δ) and 3 diabetic patients' sera (■, ●, ▲). **(b)** IgG isolated from one normal human serum (□) and 3 diabetic patients (■, ●, ▲).

TABLE 10

Binding of anti-glycated HSA and anti-ROS-glycated HSA antibodies to serum isolated proteins from normal and diabetic patients' sera

Serum	**Maximum percent inhibition at 20 µg/ml**	
Isolated Proteins	Glycated HSA	ROS-Glycated HSA
Serum Albumin		
Diabetic Patients		
1	51.7	45.0
2	47.4	50.0
3	62.9	53.2
Normal Subject	27.0	22.0
Human Immunoglobulin G		
Diabetic Patients		
1	45.8	40.3
2	47.0	45.0
3	53.0	49.0
Normal Subject	19.0	12.5

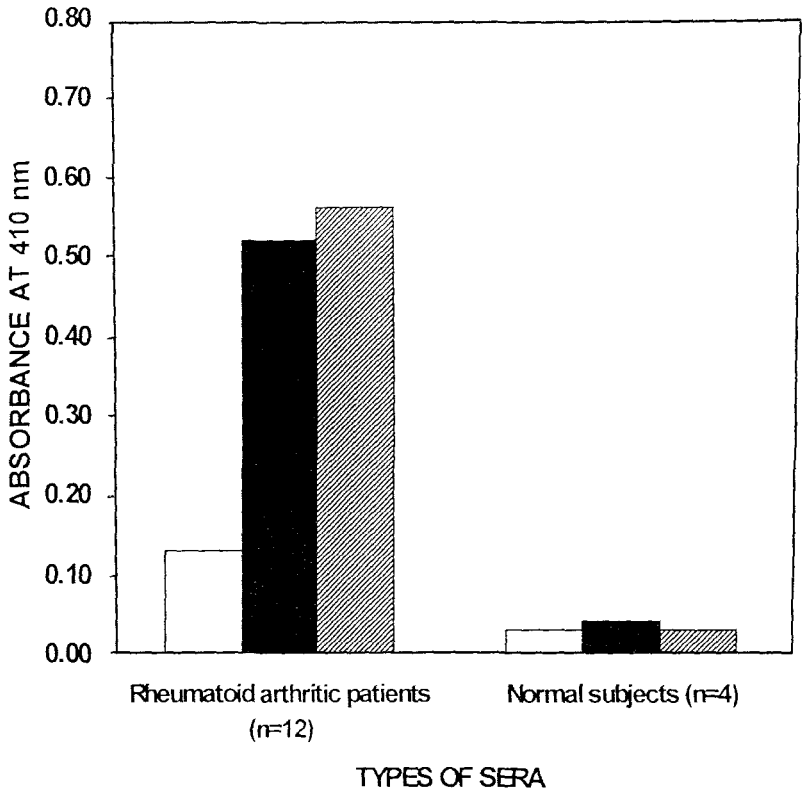


Fig. 68. Binding of rheumatoid arthritic patients' sera to native HSA (□), glycated HSA (■) and ROS-glycated HSA (▨). Normal human sera serve as control. The histogram shows the mean absorbance values of the normal and the rheumatoid arthritic patients' sera. Microtitre plates were coated with 20 μg/ml of respective antigen.

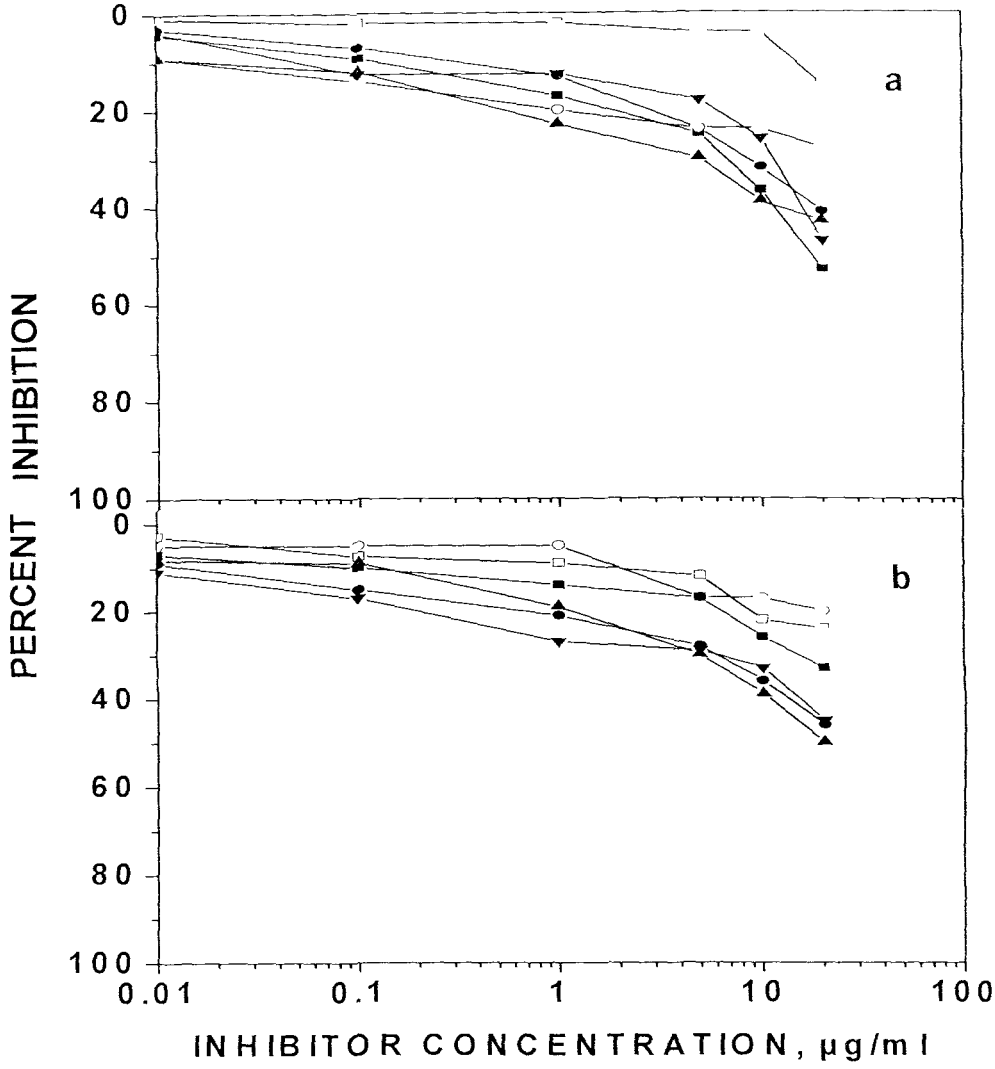


Fig. 69. Inhibition of rheumatoid arthritic autoantibodies binding by native, glycated and ROS-glycated HSA. **(a)** Rheumatoid arthritic sera 1 and 2 by native HSA (o, □), glycated HSA (●, ■) and ROS-glycated HSA (▲, ▼), **(b)** rheumatoid arthritic sera 3, 4 by native HSA (o, □), glycated HSA (●, ■) and ROS-glycated HSA (▲, ▼). The microtitre plates were coated with respective antigens (20 µg/ml).

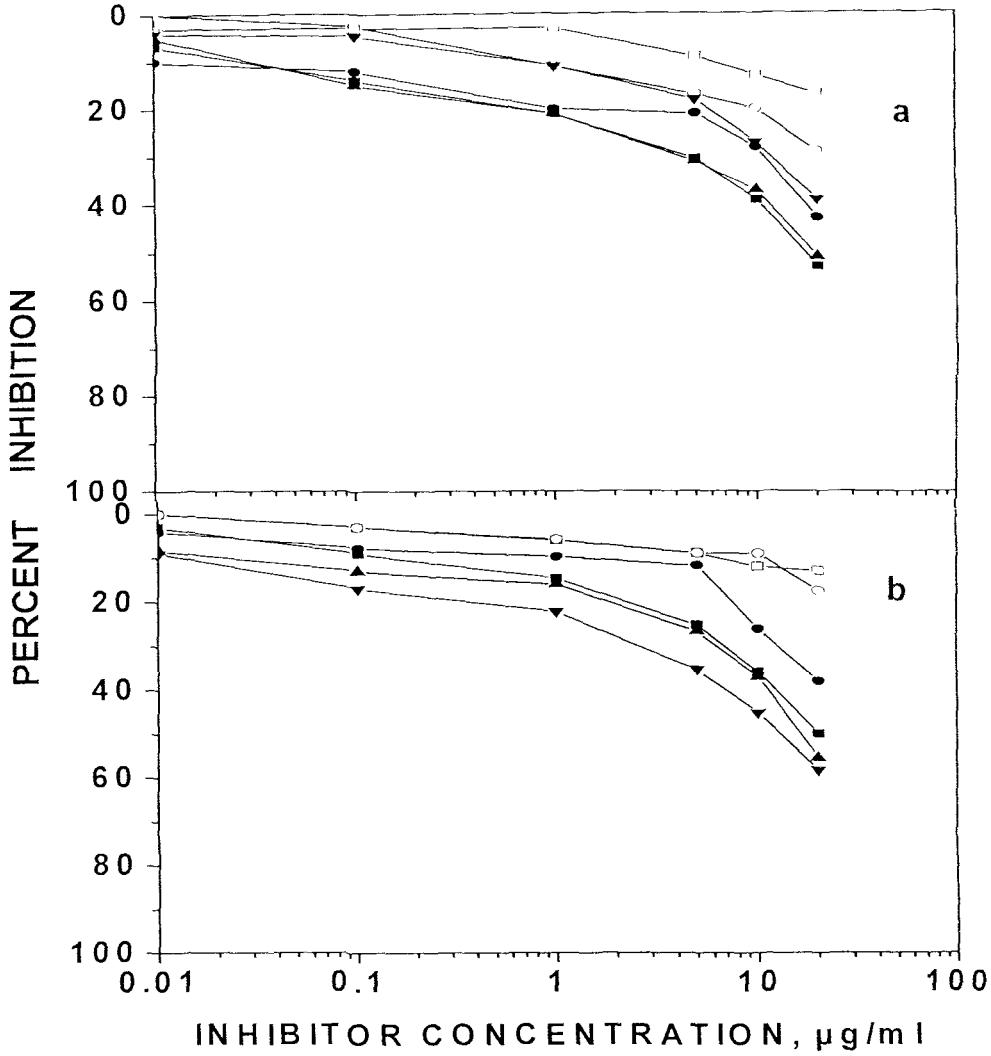


Fig. 70. Inhibition of rheumatoid arthritic autoantibodies binding by native HSA, glycated HSA and ROS-glycated HSA. **(a)** Rheumatoid arthritic sera 5 and 6 by native HSA (o, □), glycated HSA (●, ■) and ROS-glycated HSA (▲, ▼), **(b)** rheumatoid arthritic sera 7, 8 by native HSA (o, □), glycated HSA (●, ■) and ROS-glycated HSA (▲, ▼). The microtitre plates were coated with respective antigens (20 µg/ml).

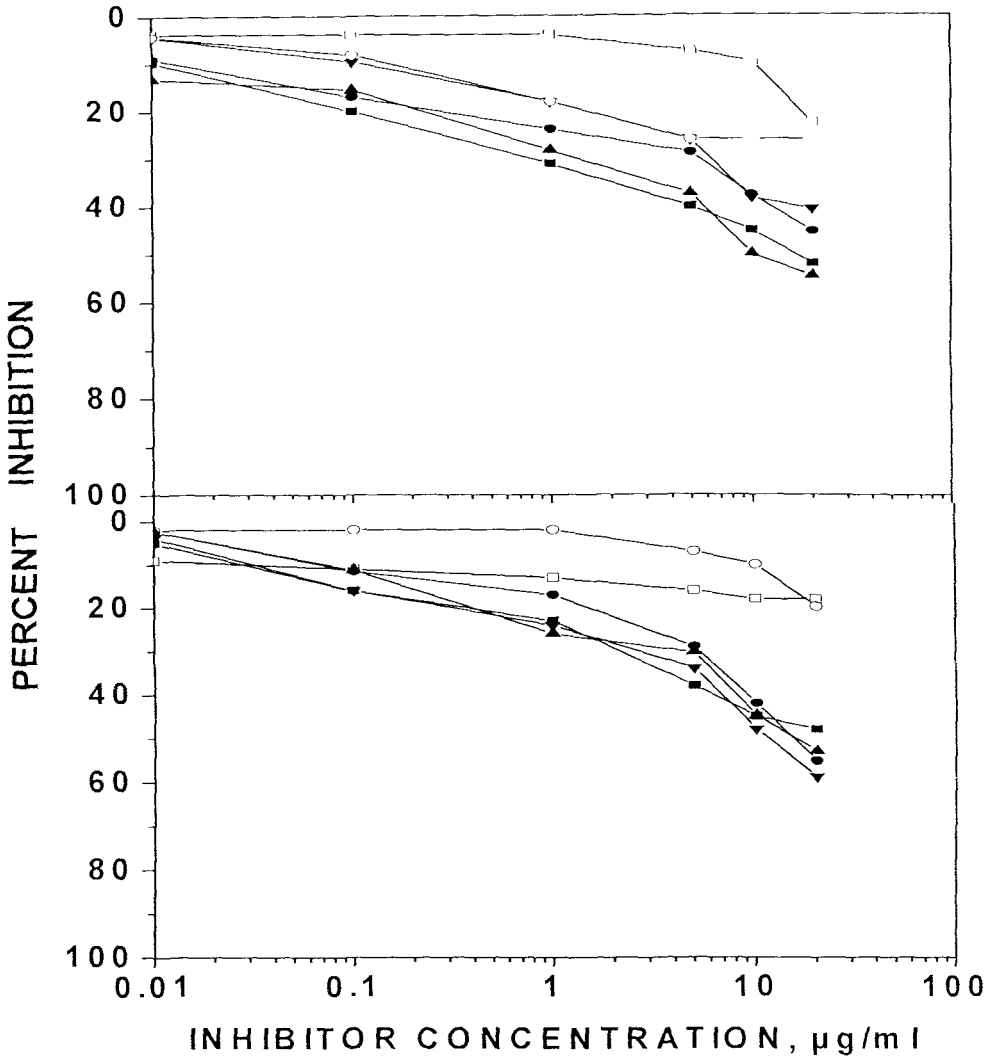


Fig. 71. Inhibition of rheumatoid arthritic autoantibodies binding by native HSA, glycated HSA and ROS-glycated HSA. **(a)** Rheumatoid arthritic sera 9 and 10 by native HSA (o, □), glycated HSA (●, ■) and ROS-glycated HSA (▲, ▼), **(b)** rheumatoid arthritic sera 11, 12 by native HSA (o, □), glycated HSA (●, ■) and ROS-glycated HSA (▲, ▼). The microtitre plates were coated with respective antigen (20 µg/ml).

Native HSA showed maximum inhibitions in the range of 13% to 29%, however, in twelve rheumatoid arthritic sera, the glycated HSA showed high percent inhibition ranging from 33% to 55.3%. Similarly ROS-glycated HSA showed still increased percent inhibition ranging from 39% to 58.8%. The inhibition data of native HSA, glycated HSA and ROS-glycated HSA with rheumatoid arthritic autoantibodies are summarized in Table 11.

Purification of rheumatoid arthritic patient IgG

Rheumatoid arthritic IgG (serum 11) was purified by affinity chromatography on Protein A-Agarose column (Fig. 72). SDS-PAGE of purified IgG under non-reducing conditions showed a single homogenous band (Fig. 72 inset).

Band Shift Assay

Band shift assay was employed to further confirm and visualize the interaction of both modified HSA samples with rheumatoid arthritis autoantibodies. A constant amount of the antigens was incubated with increasing amounts (10-40 μg) of rheumatoid arthritis IgG (serum 11) for 2 hr at 37°C and overnight at 4°C. These immune complexes were then electrophoresed on 5.5% polyacrylamide gel for 3 hr at 50 mV. Figure 73 shows the binding of rheumatoid arthritis autoantibodies to glycated HSA. Figure 74 shows the binding of rheumatoid arthritis autoantibodies to ROS-glycated HSA. With increasing concentrations of IgG there is corresponding increase in the formation of high molecular weight immune complexes which resulted in observable increase in the intensity of immune complex and corresponding decrease in the intensity of unbound antigen.

TABLE 11

Antibodies against native HSA, glycated HSA and ROS-glycated HSA in rheumatoid arthritic patients' sera

	Maximum percent inhibition at 20 µg/ml		
Sera No.	Native HSA	Glycated HSA	ROS-Glycated HSA
1	15.0	41.0	53.1
2	28.0	47.0	43.0
3	24.0	46.0	50.0
4	20.0	33.0	45.0
5	29.1	42.9	48.2
6	17.0	51.0	39.0
7	13.0	38.0	58.3
8	17.3	50.0	55.6
9	22.6	52.0	40.6
10	26.0	45.2	54.6
11	18.0	55.3	58.8
12	19.2	48.0	53.0
Mean ± SD	20.7 ± 5.2	45.8 ± 6.3	49.9 ± 6.8
(%CV)	(25.1)	(13.8)	(13.6)

ELISA plates were coated with respective antigens.
Statistically significant binding with both modified HSA ($p<0.001$) than native HSA. Values in paranthesis indicate percent of coefficient of variation.

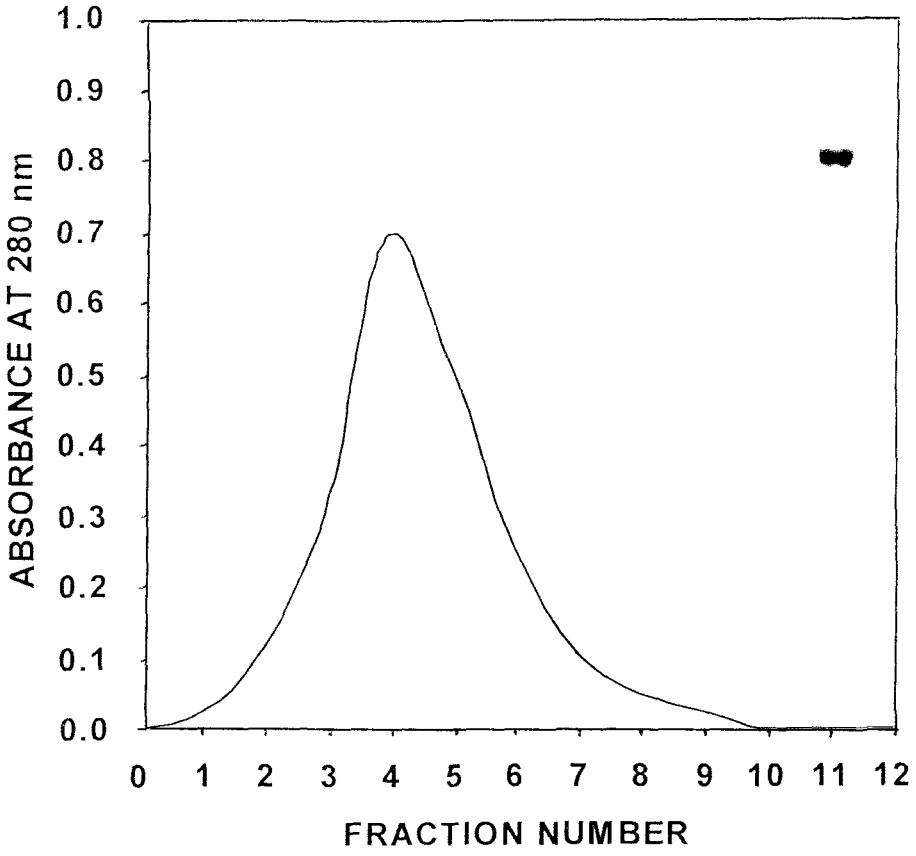


Fig. 72. Elution profile of rheumatoid arthritic patient's IgG (serum 11) on Protein A-Agarose affinity column. **Inset:** SDS-PAGE of purified IgG on 7.5% polyacrylamide gel.

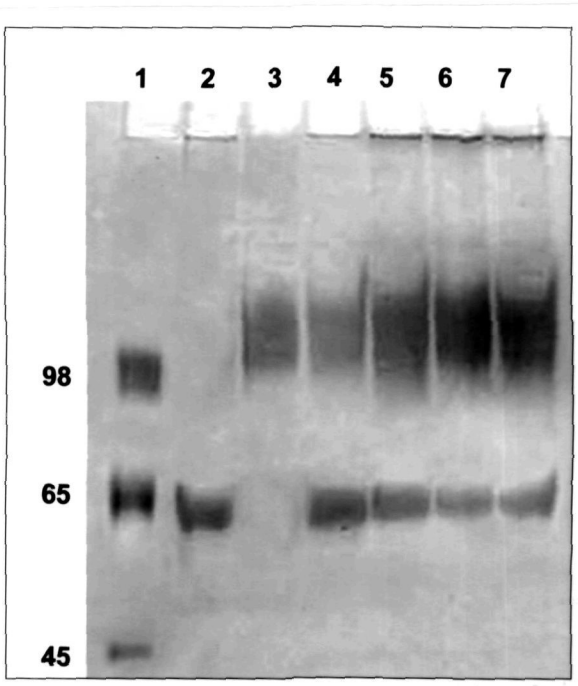


Fig. 73. Band shift assay of rheumatoid arthritic patient's IgG (serum 11) binding to glycated HSA. Glycated HSA (10 μg) (lane 2) and rheumatoid arthritis patient IgG (10 μg) (lane 3) were incubated with buffer alone. Increasing amounts of patient IgG (10, 20, 30 and 40 μg) with constant amount (10 μg) of glycated HSA through lanes 4 to 7, respectively were incubated for 2 hr at 37°C and overnight at 4°C. Lane 1 contain protein molecular weight marker (98–45 kDa). Electrophoresis was carried out on 5.5% polyacrylamide gel for 3 hr at 50 V.

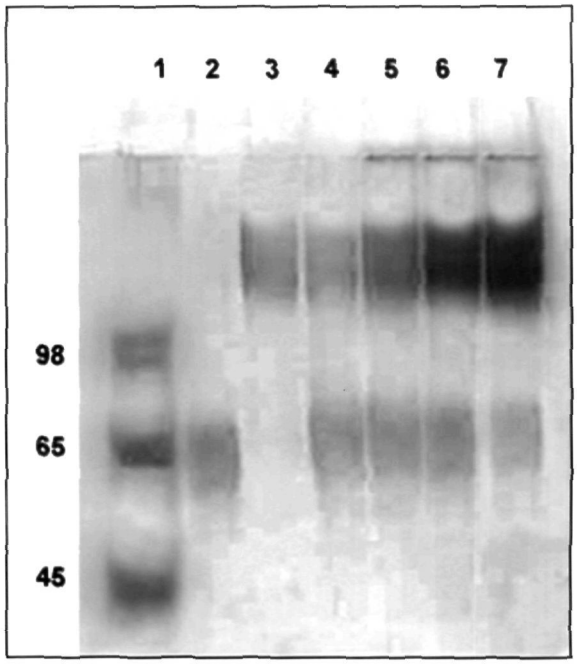


Fig. 74. Band shift assay of rheumatoid arthritic patient's IgG (serum 11) binding to ROS-glycated HSA. ROS-glycated HSA (10 μg) (lane 2) and rheumatoid arthritic patient IgG (10 μg) (lane 3) were incubated with buffer alone. Increasing amounts of patient IgG (10, 20, 30 and 40 μg) with constant amount (10 μg) of ROS-glycated HSA through lanes 4 to 7, respectively were incubated for 2 hr at 37°C and overnight at 4°C. Lane 1 contains protein molecular weight markers (98–45 kDa). Electrophoresis was carried out on 5.5% polyacrylamide gel for 3 hr at 50 V.

DISCUSSION

Non-enzymatyic glycation and oxidation play an important role in the pathogenesis of several diseases like diabetes and rheumatoid arthritis (Newkirk *et al.*, 2003; Jakus, 2003; Schmitt *et al.*, 2005). They also induce the accelerated accumulation of AGE products in tissues of diabetic patient, particularly with secondary complications like retinopathy, nephropathy and artherosclerosis (Cohen *et al.*, 2005; Defraigne, 2005). Hyperglycemia plays an important role in the pathogenesis of diabetic complications by increasing protein glycation with the gradual build up of advanced glycation end-products in body tissues. Protein glycation and the formation of AGEs are accompanied by increased free radical activity, that contributes toward the biomolecular damage in diabetes (Ahmed, 2005).

AGE formation is an inevitable process *in vivo* and can be accelerated under pathological conditions such as oxidative stress. Oxidative stress and oxidative damage to tissues are common end points of chronic diseases such as atherosclerosis, diabetes and rheumatoid arthritis. The increase in glycoxidation products in plasma and tissue proteins suggests that oxidative stress is increased in diabetes (Baynes and Thorpe, 1999; Ahmed *et al.*, 2005). In serum and synovial fluid of patients with rheumatoid arthritis, high AGE levels, like pentosidine have been found (Senolt *et al.*, 2005). In rheumatoid arthritis the generation of AGEs can be increased under oxidative stress (Drinda *et al.*, 2005; Sunahori *et al.*, 2006). The AGE carboxymethyl lysine, was observed to be present on macrophages and T cells, suggesting its role in the pathogenesis of rheumatoid arthritis (Drinda *et al.*, 2002).

Preliminary data in the present study indicates an almost exclusive formation of glycated HSA on covalent linkage of free amino groups to glucose. Glycated HSA was further modified by hydroxyl radicals ($^{\bullet}OH$), generated by irradiation of hydrogen peroxide with UV light at 254 nm. The conformational perturbation in HSA, generated on modifications, was determined by highly sensitive physico-chemical techniques such as polyacrylamide gel electrophoresis, spectral analysis, circular dichroism spectropolarimetry, thermal denaturation and formation of ketoamine, carbonyl group and free amino group. The SDS-polyacrylamide gel electrophoresis pattern of glycated HSA showed an

increase in the band intensity as well as broadening of the band towards high and low molecular weights, which can be attributed to extensive inter- and intramolecular cross-linking due to glycation, resulting in the formation of aggregates (Bouma *et al.*, 2003). However, the ROS-glycated HSA showed low intensity of band perhaps due to the fragmentation of protein into small polypeptides by the oxidation with $^{\bullet}OH$ radicals which cauld not be seen in the polyacrylamide gel.

UV-absorption spectra of glycated HSA showed substantial hypochromicity at λ_{max}. However, hyperchromicity was observed in ROS-glycated HSA. There was no apparent shift in λ_{max} for both the modified HSA. Glycation may have caused masking of aromatic amino acids due to aggregation of the protein leading to a subsequent decrease in the UV intensity. On the other hand, hyperchromicity was observed in ROS-glycated HSA either due to unfolding of the aggregates on ROS modification or degradation of polypeptide into smaller peptides accompanied by exposure of aromatic amino acids to the polar environment.

Fluorescence spectra of glycated HSA depicted a decrease in the fluorescence intensity as compared to the native HSA indicating some environmental changes around the chromophobic groups and or masking of these groups due to the formation of aggregates causes its shielding (Traverso *et al.*, 1997). Whereas, increased intensity of fluorescence was observed in ROS-glycated HSA. ROS can degrade the aggregates of the glycated HSA and chromophobic groups become more accessible to the polar environment.

HSA has a unique property in that it contains only one tryptophan residue taking this into consideration tryptophan specific fluorescence of native and modified HSA was investigated. The inhibition in the intensity of tryptophan specific fluorescence and its quantum yield after glycation were observed. ROS-glycated HSA showed increase tryptophan specific fluorescence and its quantum yield. Interestingly both modified samples showed blue shifts of 20 and 9 mm for glycated and ROS-glycated HSA, respectively, indicating that hydrophobic groups might be slightly more buried as compared to native HSA. Tryptophan specific fluorescence spectra of modified HSA and its quantum yields can be

ascribed to the destruction of the residue and/or modification of the tryptophan microenvironment (Shaklai *et al.*, 1984; Vivan *et al.*, 2001).

For further analysis of conformational and secondary structural changes in native and modified HSA, circular dichroism spectropolarimentry was carried out. In the spectral region of 200-250 nm, the CD signals of proteins are obtained mainly due to the secondary structure (Hennessay *et al.*, 1981; Manavalan, 1983). Both the modified samples showed decrease in α-helix and increase in the β-sheet structure as compared to native their forms. Random coils and turns in the secondary structure were also found to increase in the glycated and ROS-glycated HSA. Thus, glycation may serve as means for conversion of α -helix to β-sheet structure in HSA, indicating that glycation results in structural transition from the folded soluble form to β-fibrils in serum albumin (Gomes *et al.*, 2005). Differences in the ellipticity within the spectral region of 250-300 nm reflects modifications in the immediate structural and electronic environments of the aromatic amino acid residues (Creighton, 1991). Moreover, the mean residual ellipticity of both modified HSA samples showed slight decrease at 260 nm, indicating the presence of less ordered tertiary structures as compared to native.

As described earlier, sugar reacts with proteins to form stable crosslinks of protein giving stable end products (Yim *et al.*, 2001). The thermal denaturation profile of glycated HSA and ROS-glycated HSA showed a net increase of 18.1°C and 8.8°C, respectively, in the Tm value as compared to unmodified form. This can be attributed to the fact that HSA has multiple positively charged amino groups which interact covalently with the negative charges of sugar residues exhibiting inter- and intramolecular crosslinking of polypeptides, thereby, enhancing conformational stability of modified HSA. However, interaction of glycated HSA with the $^{\bullet}OH$ radicals may caused the disruption of weak bonds (hydrogen bonds) or fragmentation of the protein leading to a decrease in the Tm value compared to its glycated form (Coussons *et al.*, 1997).

The reaction with NBT is a standard method for the detection of formed ketoamines, which allowed to characterize the binding of glucose to the primary amino groups on the HSA molecules (Ahmed *et al.*, 2002). It was found that the ketoamine generation in glycated HSA was significantly higher than its native

form. ROS-glycated HSA showed slightly lower levels of ketoamine as compared to glycated form of HSA. Ketoamines are early non-enzymatic glycation adducts and are important precursors of AGEs and hydroxyl radicals which can disrupt the sugar-protein binding resulting in decrease of ketoamine level on ROS modification (Ahmed *et al.*, 2002).

Ketoamines were converted to protein bound carbonyl compounds via a protein enediol reaction (Baynes, 1991). The generation of carbonyl groups serve as a marker of protein glycoxidation (Bayens and Thorpe, 1999). Modified HSA exhibited significant amounts of carbonyl groups, whereas, unmodified HSA showed negligible amounts. Moreover, ROS glycated HSA contained the highest level of carbonyl groups bound to protein.

Number of free amino groups of proteins irreversibly modified due to non-enzymatic glycation (Traverso *et al.*, 1997). The observed number of free amino groups in the glycated HSA reduced to half the in number present in its native form, whereas, ROS-glycated HSA showed noteworthy high number of free amino groups per molecule of HSA as compared to native HSA. The increased number of free amino groups in ROS-glycated HSA may be attributed to the $^{\cdot}OH$ radical modification which lead to the generation of new amino groups by polypeptide fragmentation (Baynes and Thorpe, 1999).

Incubation of modified HSA samples with metal chelators, free radical scavengers and antioxidants showed inhibition in the glucose and ROS modification (Giardino *et al.*, 1998). Metal chelators can sequester the metal ions and hence given respectable inhibition in the glycation (Thornalley *et al.*, 1999). Superoxide and hydroxyl radical are generated during glycation via Fenton reaction (Hunt *et al.*, 1988). Non-enzymatic antioxidants sodium azide, mannitol, ascorbic acid and enzymatic antioxidant SOD and catalase showed inhibition in glucose modification The addition of non-enzymatic and enzymatic antioxidants prevented AGE-induced damage, suggesting an enhanced formation of reactive oxygen species. Aminoguanidine is a potent inhibitor of glycation and exhibited maximum inhibition in glucose modification (Singh *et al.*, 2001).

Almost similar results were obtained with metal chelators and showed considerable inhibition in ROS modification. Mannitol is a specific quencher of

$^{\bullet}OH$, induced remarkable inhibition in modification. Whereas, sodium azide showed least inhibition. Ascorbic acid and SOD exhibited similar inhibitions. Potent inhibition was observed by catalase and combination of catalase and SOD indicating the generation of hydrogen peroxide and some amount of superoxide during modification. The above results revealed that in ROS modification, maximum formation of $^{\bullet}OH$ radical occur, however, less amounts of $^{-\bullet}O_2$ were generated. These results also showed the formation/presence of H_2O_2 (Yim *et al.*, 2001).

Antibodies against native and modified HSA were induced in rabbits by immunization with native, glycated and ROS-glycated HSA which were subsequently evaluated by direct binding, immunodiffusion and inhibition assay. Both modified HSA showed higher antibody titer than the native form. The antigenic specificity of purified IgG of native and modified HSA was ascertained by competition and band shift assay. The results revealed a high affinity of native and modified IgG for their immunogen, suggesting that the induced antibodies are immunogen specific and probably recognizing the modified structure or conformation of modified HSA samples.

From the observed data, the modified HSA was found to be more immunogenic than its native form in the experimental animals. This could be attributed to the feature of hyperglycemia and inflammatory disease rheumatoid arthritis wherein, glucose and ROS modification play an major role in the production of autoantibodies, thus generating new potential epitopes against which the antibodies were raised. Autoantibody targeted against intracellular are proteins serological hallmarks of the diabetes mellitus and rheumatoid arthritis (Mikino *et al.*, 1995; Sunahori *et al.*, 2006). Antiglycated HSA antibodies are significantly high in the diabetics than in control subjects (Schalkwijk *et al.*, 1999), moreover, AGE damaged IgG can be detected in patients with rheumatoid arthritis (Pullerits *et al.*, 2005).

Glycated forms of IgG, BSA, poly-L lysine, HSA (20 weeks) and fructated HSA showed significant inhibition in the range of 43% to 58.6% with anti-glycated HSA antibodies. These results demonstrated the preferential recognition of glucose modified protein by anti-glycated antibody, providing

convincing evidence for the formation of common epitopes on protein glycation. Anti-ROS-glycated HSA antibodies showed remarkable inhibition (38.9%-55%) with the ROS modified glycated protein inhibitors (ROS-glycated IgG, ROS-glycated BSA, ROS-glycated poly-L lysine) and ROS-HSA which suggests an appreciable binding of ROS modified proteins to the anti-ROS glycated HSA antibodies responsible for the generation of common epitopes on ROS modification. However, native forms of all above mentioned protein inhibitors did not show significant binding towards anti-modified HSA antibodies. Native plasmid DNA did not show reactivity with either of the antigen. From the data, it was predicted that new epitopes have been developed during glycation and subsequent ROS modification. From the cross reactivity experiments it was concluded that modified HSA antibodies are polyspecific and cross reacting with variety of inhibitors recognizing comman antigenic determinants or altered protein conformation.

AGEs tend to accumulate in the tissues of diabetic patients (Kume *et al.*, 2005). Protein containing AGEs are highly immunogenic and are recognized by anti-AGE antibodies (Reddy *et al.*, 1995; Ikeda *et al.*, 1996). Immunoglobulin G reactivity towards AGE-HSA was significantly higher in diabetic than in control sera (Vay *et al.*, 2000). Increased production of free radicals by mitochondria induced by hyperglycaemia may be responsible for the observed metabolic disturbances (Defraigne, 2005).

In the present study the possible role of native and modified HSA in diabetes was investigated. Twenty four sera of diabetic patients, showing high titre with glycated HSA and ROS-glycated HSA, were collected for the study. These sera showed higher titer with modified HSA than with native polymer as ascertained by competition ELISA. The inhibition observed in the order of ROS-glycated HSA > glycated HSA > native HSA. These results indicate that the modified HSA is an effective inhibitor, showing substantial difference in the recognition than in its native form. Band shift assay further sustained the binding of glycated and ROS-glycated HSA with IgG of diabetic patients. The strong binding of diabetic patients' sera IgG towards modified HSA demonstrates the possible role of modified HSA in diabetic pathogenesis.

Role of native and modified HSA was also probed in the pathogenesis of diabetic patients having secondary complications (retinopathy, nephropathy and artherosclerosis). Earlier studies have shown that diabetic patients with secondary complications indicate the presence of the autoantibodies in the plasma against AGEs (Rahbar and Figarola, 2003). Further more, the autoantibodies against AGEs were higher in the patients with the renal failure than in normal subjects or diabetic subjects without renal failure (Shibayama *et al.*, 1999). Diabetic individuals may exhibit elevated levels of iron and free copper ions (Cutler, 1978: Mateo *et al.*, 1978), which in the presence of glycated proteins have been shown to generate free radicals (Hunt, 1994). These highly reactive species may cause oxidative degradation of serum proteins and this effect has been used as a potential explaination for diabetic secondary complications. In our studies maximum recognition of ROS-glycated HSA was observed in sera of diabetic retinopathic patients followed by the recognition of glycated HSA. Similarly high recognition of modified polymers was observed with the sera of nephropathic and artherosclerotic diabetic patients. However, no remarkable inhibition was observed in normal human sera for both modified antigens.

The level of serum AGEs could be considered as a marker of later development of secondary complications in both types of diabetes mellitus (Defraigne, 2005). Recently it was found that glucose could probably oxidize to form reactive carbonyl compounds, which may react with protein to give glycoxidation products (Valencia *et al.*, 2004). Immunohistochemical studies using anti-AGE antibodies have revealed the presence of AGE modified proteins in several tissues under various pathological conditions (Mikino *et al.*, 1995).

Apart from albumin, the other serum proteins, including immunoglobulins and red blood cells are also glycated in diabetic plasma (Myint *et al.*, 1995). Albumin isolated from three diabetic patients recognizes anti-glycated and anti-ROS-glycated HSA antibodies. IgG isolated from same number of diabetic patients recognized anti-glycated and anti-ROS-glycated HSA antibodies. Whereas, albumin and IgG from normal human sera showed no remarkable inhibition. Hence our result is in agreement with the earlier studies that glycated serum proteins are more toxic than their native form (Bouma *et al.*, 2003).

The presence of autoantibodies reactive towards native and modified HSA in the sera of patients with rheumatoid arthritis has also been studied. The study consisted of 12 sera from patients with sero positive and negative. All the sera showed higher reactivity towards modified HSA. Increased levels of circulating autoantibodies have been reported in sera of patients with rheumatoid arthritis than the normal subjects (Drinda *et al.*, 2002). The data presented here clearly indicate the presence of circulating antibodies in rheumatoid arthritic sera to glycated HSA and ROS-glycated HSA, however, native HSA showed less reactivity towards the rheumatoid arthritic sera. It has been well accepted that glycation and glycoxidation to protein play a fundamental role in the pathogenesis of rheumatoid arthritis (Sunahori *et al.*, 2006). Band shift assay further substantiated the binding of glycated HSA and ROS glycated HSA with the circulating antibody in rheumatoid arthritic sera.

The data clearly supports the notion that glucose modified-HSA and subsequent ROS attack lead to the generation of circulating autoantibodies in rheumatoid arthritic patients.

Based on the above studies the following conclusions can be drawn:

1. Incubation of HSA with glucose resulted in the formation of early and advanced glycation end products.
2. Glycated HSA was further modified by ROS generated by 254 nm irradiating in the presence of hydrogen peroxide.
3. Both the modifications cause major biochemical and biophysical changes in HSA.
4. Native and modified HSA were found immunogenic in experimental animals. However, modified HSA are highly immunogenic as compared to native HSA.
5. Induced antibodies of modified HSA were higly specific for the immunogens and also showed cross reactivity with glycated and ROS-

glycated form of inhibitor proteins, showing polyspecificity.

6. Diabetic autoantibodies recognized the glycated and ROS-glycated HSA better than the unmodified form. Moreover, the diabetic patients with secondary complications showed higher binding to modified HSA than diabetic and normal subjects.

7. Serum isolated proteins also exhibited significant reactivity towards the anti-glycated and anti-ROS-glycated HSA antibodies.

8. Antibodies in the sera of various rheumatoid arthritic patients were found to be more specific for modified antigens than their unmodified analogue.

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